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**Mechanisms of immunomodulation by *Brugia pahangi*  
infective larvae and microfilariae**

Julie Osborne

A thesis submitted for the degree of  
Doctor of Philosophy

Department of Veterinary Parasitology,  
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University of Glasgow

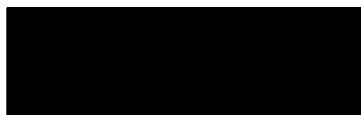
February 1997

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## DECLARATION

The author was responsible for all the experiments carried out in this thesis, except where it is otherwise stated. No part of this work has been submitted for any other degree but has been reproduced in part in the following publication:

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## SUMMARY

Lymphatic filariasis in humans is characterised by a profound bias in the immune response. Parasite specific Th1 responses, including proliferation, are dramatically impaired while Th2 responses predominate. In this study, a mouse model of filariasis was used to investigate the role of the infective form (the third stage larvae, L3) and the blood stage form (the microfilariae, mf) in modulating the immune response. Subcutaneous infection of BALB/c mice with L3 and mf of *Brugia pahangi* has a profound and contrasting effect on Th cell function, that appears to replicate, at least in part, the two striking aspects of the human immune response

A Th2 response predominated and polyclonal Th1 responses and antigen-specific proliferation are down-regulated in L3-infected mice. Surprisingly, antigen-specific proliferation was absent in mf-infected mice in which a Th1-biased response dominated. Furthermore, after four days of culture in the presence of antigen spleen cells from mf-infected mice, but not L3-infected mice or uninfected controls, displayed a S.I.<1. These data would suggest that spleen cells primed by infection with mf are undergoing accelerated death in culture. The remaining part of the study was focused on examining the mechanisms underlying the skewed responses in both L3- and mf-infected animals that may suggest some novel pathways operating in the infected human.

Treatment of spleen cells from L3-infected mice with neutralising anti-IL-4, anti-IL-10 or rIL-2 resulted in a dramatic increase in ConA-driven proliferation, IL-2 and IFN- $\gamma$  production. Interestingly, removal of the resident spleen APC population and replacement with APC from uninfected animals also restored the defective mitogen-driven Th1 responses. Furthermore, replacing the APC population or neutralising IL-10, but not IL-4, resulted in antigen-specific IL-2 and IFN- $\gamma$  indicating that *B. pahangi*-primed Th1 cells do exist in L3-infected mice but appear to be unable to respond in the presence of IL-10 perhaps operating via its effect on APC function.

The early cytokine transcription pattern elicited by infection with L3 and mf was also analyzed in parallel. At 24h post-infection (p.i.) a burst of IL-4 transcription was detected in the draining popliteal lymph node of L3 infected animals. IL-4 was the only cytokine transcript detectable at this early time point and was not present in mf-infected mice. From day 4 p.i. onwards, the L3 elicited a Th2 response as defined at the level of cytokine mRNA and protein production by CD4<sup>+</sup> cells. In contrast, mf stimulate high levels of IFN- $\gamma$  mRNA at day 4 p.i. in the absence of IL-4 or IL-10 induction. Cell selection analysis indicated that the IL-4 produced at 24h derived from a population of CD4<sup>-</sup> CD8<sup>-</sup> T cells. Further analysis using antibodies to the  $\gamma\delta$  and  $\alpha\beta$  TCR demonstrated that  $\gamma\delta$  T cells are not likely to contribute to the early burst of IL-4. These

results suggest that triggering of an unusual double negative T cell population to secrete IL-4 at the very outset of infection with the L3 of *B. pahangi* may be the critical factor favouring the development of antigen-specific Th2 cells in response to this stage of the parasite.

The aim of the final part of the study was to investigate whether apoptotic cells could be detected in the spleens from mf-infected mice. The results of these experiments demonstrated that freshly isolated spleen cells from mf-infected mice did indeed contain apoptotic cells as evidenced by a significant proportion of hypodiploid lymphocytes and fragmented DNA. In contrast, freshly isolated spleen cells from L3-infected and uninfected control mice did not contain hypodiploid cells and the DNA prepared from those cells was intact. Consistent with these results spleen cells sections from mice injected s.c. or i.v. with mf, but not s.c. with L3 or HBSS, displayed a distinctive pattern of TUNEL-stained clusters of cells within the majority of follicles.



## LIST OF ABBREVIATIONS

aa	amino acid
APC	antigen presenting cell
BSA	bovine serum albumin
Ci	Curie
CO <sub>2</sub>	carbon dioxide
cpm	counts per minute
dCTP	deoxycytidine triphosphate
ddH <sub>2</sub> O	double distilled water
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunoabsorbent assay
FACS	fluorescent activated cell sorting
FITC	fluorescein isothiocyanate
FCS	foetal calf serum
g	gram
HCl	hydrochloric acid
HRPO	horseradish peroxidase enzyme
iNOS	inducible nitric oxide synthase
KCl	potassium chloride
KH <sub>2</sub> PO <sub>4</sub>	potassium dihydrogen phosphate
λ	lamda
l	litre
L3	infective larvae
M	molar
MAb	monoclonal antibody

mA milliamp  
 $\mu$ Ci micro Curie  
mCi milli Curie  
 $\mu$ g microgram  
mg milligram  
 $\mu$ g microlitre  
ml millilitre  
 $\mu$ m micrometre  
 $\mu$ M micromolar  
mM millimolar  
mRNA messenger RNA  
mf microfilariae  
MW molecular weight  
NaCl sodium chloride  
NaHCO<sub>3</sub> sodium bicarbonate  
Na<sub>2</sub>HPO<sub>4</sub> di-sodium hydrogen phosphate  
NaOH sodium hydroxide  
NH<sub>4</sub>Cl ammonium chloride  
NO nitric oxide  
°C degrees centigrade  
OD 620nm optical density at wavelength 620nm  
PCR polymerase chain reaction  
PBS phosphate buffered saline  
RNA ribonucleic acid  
RNase ribonuclease  
rpm revolutions per minute  
RT reverse transcriptase  
SD standard deviation  
SDS sodium dodecyl sulphate

SEB staphylococcal enterotoxin B

TAE Tris-Acetate EDTA buffer

TBE Tris-Borate buffer

TMB 3,3',5,5'-tetramethylbenzidine

Tris Tris (hydroxymethyl) methylamine

U unit

V volts

v/v volume per volume

w/v weight per volume

# **CHAPTER 1**

## Chapter 1 Introduction

### 1. The Parasite

#### 1.1. The Parasites

Lymphatic filariasis is a serious and debilitating disease of man. Three species of nematode parasites are responsible: *Wuchereria bancrofti*, *Brugia malayi* and *B. timori*. Of the estimated 118 million people infected, 90% (105 million) have *W. bancrofti* and less than 10% (13 million) have *B. malayi* infections (Michael *et al*, 1996). *B. timori* has been detected only in the Indonesian Islands of Timor, Flores, Rote and Alor. *W. bancrofti* is found world-wide, throughout the tropics, while *B. malayi* is restricted to South-East Asia, China and Korea. About two-thirds of those infected with lymphatic filariasis live in China, India and Indonesia.

#### 1.2. Life History

Lymphatic filarial worms, like other nematodes, have five developmental stages in their life cycles. The life cycle is indirect, involving an obligatory period within a mosquito host. *Aedes*, *Anopheles*, *Culex* and *Mansonia* mosquitoes are the vectors of lymphatic filariae. Microfilariae (first stage larvae [mf]), measuring only 210x6 µm, circulate in the blood and are ingested by feeding mosquitoes. Inside the mosquito the mf penetrate through the wall of the midgut into the haemocoel. From there they invade the cells of the thoracic muscles and moult twice to become third stage (infective) larvae (1.5x0.03 mm) which then migrate mainly to the mouthparts. When the mosquito bites the human, the L3 emerge from the mouthparts and enter the skin, apparently through the wound made by the proboscis (Denham and McGreevy, 1977). The larvae migrate to the lymphatics where they moult twice to become adolescent and then fully mature adults that reside primarily in the afferent lymphatic vessels. The adult worms are white and thread-like in appearance and the females are approximately double the size of males. Female and male *B. malayi* worms measure 40x1.5 mm and 22x1 mm

respectively. *W. bancrofti* adults are larger; females and males measure 90x3 mm and 40x1.5 mm respectively. After mating the females lay mf that circulate in the bloodstream to be ingested in the next mosquito blood-meal and continue the life cycle.

Throughout most of its range *W. bancrofti* is nocturnally periodic. It is transmitted mainly by *Culex quinquefasciatus* a domesticated, night-biting urban mosquito. The prevalence of periodic *W. bancrofti* is correlated with density of population and poor sanitation since *C. quinquefasciatus* breeds mainly in water contaminated with sewage and decaying organic matter. In rural areas it can be transmitted by *Aedes* spp. and *Anopheles* spp. In contrast, in the definitive host *W. bancrofti* is remarkably host specific in that natural infections only occur in humans (*In Basic Clinical Parasitology*, 1993).

In most of its endemic area *B. malayi* is nocturnally periodic and is found mainly in agricultural areas where anopheline and aedine vectors predominate and bite only at night. It can also be transmitted by *Mansonia* spp. Subperiodic *B. malayi* is considered to be a zoonosis with monkey, cat and dog reservoirs. *Mansonia* mosquitoes feed on a variety of animal hosts including man, cats, dogs and monkeys and subperiodic *B. malayi* infections occur naturally in domestic carnivores and are common in wild monkeys. This animal reservoir has important implications for parasite control. In contrast, periodic *B. malayi* is thought to be contained almost exclusively in the human population (Mak, 1986).

*B. timori* is nocturnally periodic and appears to be limited to the human population of a few Indonesian Islands where it is transmitted by *Anopheles barbirstris*. *B. pahangi* is a common parasite of wild and domestic animals Southeast Asia. Although isolated incidences of experimental (Edeson *et al*, 1960) and natural (Palmieri *et al*, 1985). *B. pahangi* infections in man have been reported, more extensive surveys have proved fruitless.

## 1.2. The Human Disease

### 1.2.1. Immunodiagnosis and Control

Lymphatic filariasis is a major cause of morbidity throughout the tropics. Infections are generally chronic and often disabling and as such are extremely detrimental to socio-economic development in much of Asia, Africa, and the Western Pacific. In addition to the 120 million people that are known to be infected, a further 900 million are considered to be at risk of infection by living in endemic areas (ref). The prevalence of the disease in rural and urban slums in tropical countries means that it predominantly affects the poorer section of the community. The incidence of disease is increasing world-wide mainly because of unplanned urbanisation of endemic areas (Ottesen and Ramachandran, 1995). The other major filarial infections of man are *Onchocerca volvulus* (20 million in West Africa and Central and South America) and *Loa Loa* (13 million in Africa) (WHO, 1984).

Currently there are two main programs to control the transmission of lymphatic filariasis. The first involves vector control to reduce human-vector contact. The second is directed at treating the human host to reduce microfilaraemia. These projects need to be implemented together for long-term effect, although differences in local requirements should dictate the most efficient overall strategy.

Reduction in vector intensity can be achieved on several levels. The destruction of breeding grounds gives permanent results but involves high initial and maintenance costs. The removal of aquatic plants using cheap herbicides has been the principal means of control of *Mansonia* sp. (the major vector of sub-periodic *B. malayi*), larvae and pupae of which acquire oxygen by attaching to the underwater root, stems and leaves of aquatic plants like *Pistia*. Insecticide spray programs to control malaria have also been shown to interrupt transmission of filariasis in areas where they co-exist.

Indoor spraying of long-lasting pyrethroids and the use of insecticide impregnated nets and bedding has been effective against *C. quinquefasciatus* and other domesticated mosquitoes. However, the emergence of resistant strains of mosquito and the ecological damage caused by the indiscriminate and widespread use of insecticides has stimulated alternative approaches to vector control. Polystyrene beads can be used to "suffocate" mosquito larvae in urban breeding sites. In addition, biocides like the toxin producing bacterium *Bacillus sphaericus* have been used to control *C. quinquefasciatus* (WHO, 1992).

For a number of years low dose treatment of microfilaraemic (mf+) individuals with the microfilaricidal drug diethylcarbamazine (DEC) has proved to be extremely effective in removing mf from the circulation, although with some side-effects (Ottesen, 1985; Partono *et al*, 1989). DEC treatment can also alleviate some of the symptoms of pathology (Partono *et al*, 1989).

It has now become apparent that mass treatment of the human population is necessary, including asymptomatic carriers who may be reluctant to seek therapy. Therefore, treatment regimes, that are more easily administered, have also been amended to minimise the side-effects that appear to be dose-dependent (Ottesen and Ramachandran, 1995). A single annual or bi-annual dose of DEC (6mg/kg body weight) reduces mf levels by 90-95% for at least one year. A similar outcome was obtained by the substitution of normal cooking salt with DEC-fortified salt (0.2%-0.4% w/w) for a period of 9-12 months. This treatment protocol is well tolerated and can be safely used during pregnancy. However, in *Onchocerca* and *Loa Loa* infections the reaction to treatment with DEC may be quite severe. This does not appear to be the case for another, more expensive microfilaricidal drug, ivermectin. As effective and safe as single dose DEC treatment for lymphatic filariasis, ivermectin is currently being developed as the drug of choice in areas where lymphatic filariasis co-exists with other filarial infections (Ottesen and Ramachandran, 1995).



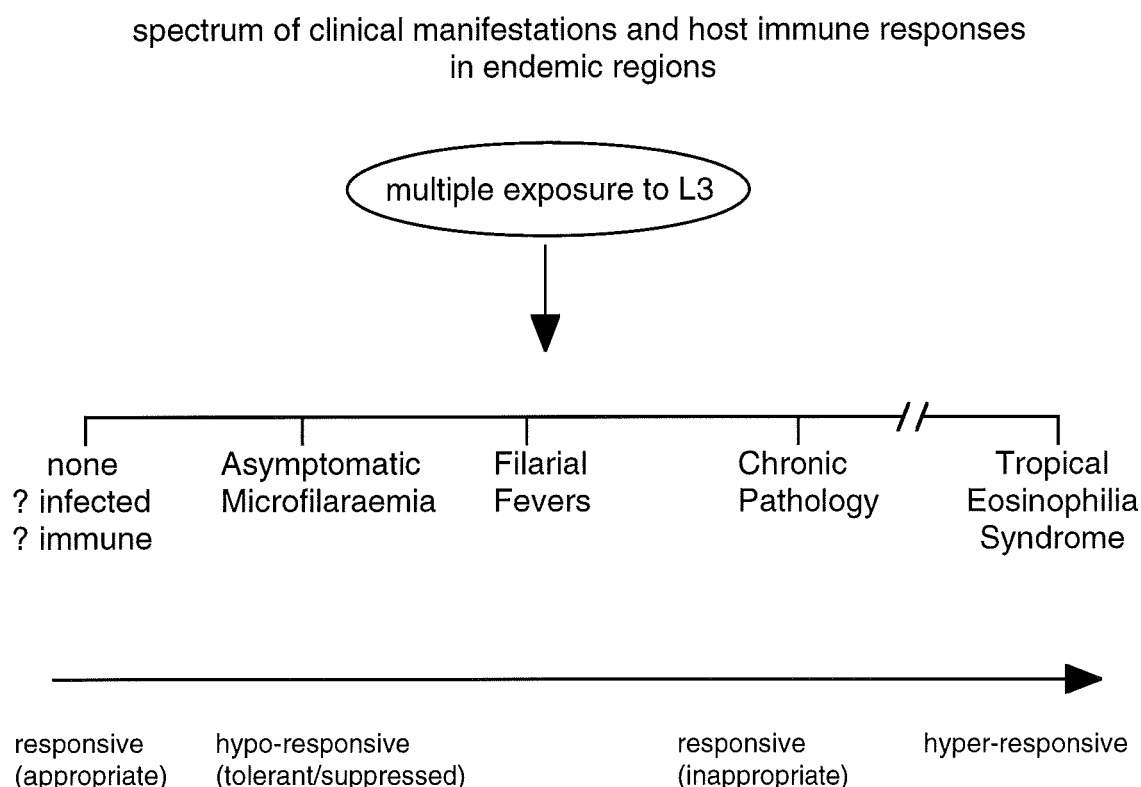
In the context of complete disease eradication, the long-term efficacy and safety of the new treatment regimes need to be assessed. Lymphatic filariasis is known to be a chronic long-term infection where individual adult worms can survive for up to five years (Vanamail *et al*, 1996). While DEC and ivermectin are efficient microfilaricides they are much less potent against adult worms (Ottesen, 1985; Day *et al*, 1991). The localised killing of adult worms has been reported following DEC treatment (Ottesen, 1985), but this in itself raises a serious dilemma since much of the lymphatic pathology in filariasis is thought to reflect severe inflammatory responses due to/or following the death of adult worms.

Diagnosis still relies upon the demonstration of mf by microscopic examination of blood samples. Membrane filtration and concentration techniques have been developed to detect low levels of microfilariae. However, an important element to studying lymphatic filariasis, and monitoring control strategies, is establishing which individuals are actively infected. This requires an assay sensitive enough to detect very low level or amicrofilaraemic infection that also permits discrimination between current and past infections. These criteria have been met by the development of assays based on the detection of parasite antigen or specific antibody. *W. bancrofti* infections can be readily screened for using a MAb to phosphorylcholine-containing antigen (PC-Ag) which functions as an indirect measure of adult worm burden (Day *et al*, 1991a). The detection of antigen-specific IgG4 is considered to be indicative of active *Brugia* infection, even in the absence of other clinical or parasitological evidence (Ottesen *et al*, 1985a; Kwan-Lim *et al*, 1990; Kurniawan *et al*, 1993). The superior sensitivity of these assays has also allowed the diagnosis of sub-clinical infections in individuals that would previously have been considered infection-free by mf counts alone (Day *et al*, 1991b). Similarly, improvements in lymphatic imaging have detected lymphatic abnormalities in infected but otherwise asymptomatic individuals. Unlike previous techniques used to visualise the lymphatics, lymphoscintigraphy can be used to assess

more accurately the effect of chemotherapeutic measures and also the level of morbidity in endemic populations (Ottesen and Ramachandran, 1995). The use of PCR based methods and species-specific DNA probes is another very sensitive method for detecting filarial infection in humans (McCarthy *et al*, 1996) and mosquito vectors (Chanteau *et al*, 1994).

### 1.2.2. The Spectrum of Infection

Lymphatic filariasis is manifested by a spectrum of symptoms that range from asymptomatic microfilaraemia to gross pathology (see below). A proportion of the adult population that are amicrofilaraemic (mf-) and have no symptoms or history of lymphatic disease have been classified as endemic normals (ENs), although this is a controversial grouping. A small number of individuals are found with the hyper-responsive syndrome called TPE.



## **Pathology:from ADL to Chronic Lymphatic Obstruction**

Filarial symptoms are caused mainly by the lymphatic dwelling adult worms, living as well as dead and degenerating, although there is evidence that opportunistic bacterial infection is a contributory factor. Initially, damaged lymphatic vessels result in lymphatic incompetence leading to lymphodema. This state is reversible with chemotherapy. Granulomatous reactions around trapped worms occludes small lymphatics and narrows the larger ones. For many patients, intermittent and painful attacks of lymphangitis and adenolymphangitis (ADL), accompanied by fever, is the only indication that they are infected since they are generally mf-. In most cases the site of lymphadenitis is the inguinal region. Following years of continuous filarial infection, and preceded by chronic oedema and repeated acute inflammatory attacks, obstructive filariasis develops. The permanent and incredibly debilitating conditions of elephantiasis and hydrocoele (in males with bancroftian filariasis) represent gross abnormalities due to lymphatic obstruction. The high protein content of the oedematous lymph stimulates the growth of dermal and collagenous connective tissue, and gradually over a period of years, the enlarged affected parts harden, producing chronic elephantiasis. In bancroftian filariasis, chyluria occurs when lymphatic vessels rupture discharging chyle into the urinary tract. The clinical disease caused by *B. timori* is very similar to that of *B. malayi* except that abscess formation is common in the early stages, while chronic lymphodema is more common than frank elephantiasis in later cases. While CP patients tend to be mf-, there is a proportion of individuals that exhibit gross pathology while remaining mf+ (see 1.2.8).

## **Tropical Pulmonary Eosinophilia (TPE)**

TPE is a rare condition caused by immunological hyper-responsiveness to filarial infection. Symptoms include very high blood eosinophil levels (>3000/ml), night coughs, wheezing, weight loss, fatigue, and interstitial lung lesions visualised by X-ray. This acute pulmonary inflammation can lead to chronic interstitial fibrosis. TPE is distinguished from similar "pulmonary infiltrates with eosinophilia" syndromes by the

very high levels of parasite-specific IgE and IgG and a rapid clinical response to microfilaricidal DEC treatment. TPE lung pathology is associated with hypersensitivity reactions induced by degenerating mf that have been trapped in the lungs following antibody opsonization.

### **Asymptomatic Microfilaraemia**

Asymptomatic microfilaraemics make up the majority of the filarial-infected endemic population. They display no overt signs of clinical disease yet can have extremely high levels of mf circulating in their blood (up to 20,000 or more mf/ml ). The inability to clear their microfilariae mean that these individuals serve as a reservoir for continued transmission of infection. In addition, asymptomatic infected subjects can suffer subclinical abnormalities of lymphatic and renal function.

### **Endemic Normals**

Despite life-long exposure to infection a proportion of the endemic population have no detectable mf and no symptoms or history of the disease. However, it is unlikely that such endemic normals (ENs) are truly infection-free. This group is likely to contain those with pre-patent or single-sex infections or very low levels of microfilariae.

### **1.2.3. Immunological Correlates of Immunity, Infection and Disease**

The diversity of clinical symptoms present in the endemic population is considered to reflect the type and intensity of the immune response mounted to the parasite (Ottesen, 1980; Ottesen, 1992). PBMCs from asymptomatic microfilaraemic individuals are generally unresponsive to filarial antigens in *in vitro* proliferation and Ab production assays. In contrast, PBMCs from patients with CP, who are normally mf-, generally exhibit increased *in vitro* T and B cellular reactivity to filarial antigens (Ottesen *et al*, 1977; Piessens *et al*, 1980; Nutman *et al*, 1987; Nutman *et al*, 1987a). TPE patients are considered to display a distinct type of hyper-reactivity while healthy ENs tend to

generate elevated parasite-specific T and B cell responses that are considered "appropriate" (Nutman *et al*, 1987a; Ottesen *et al*, 1982; Gallin *et al*, 1988).

The paradoxical relationship between parasite density, in terms of circulating microfilariae, and host reactivity and disease is an intriguing feature of lymphatic filariasis. Since most infected individuals are long-term asymptomatic microfilaraemics this balance is considered to reflect a highly evolved host-parasite relationship. It has been hypothesised that the parasites (adults and/or microfilariae) induce a form of immunological unresponsiveness that prevents parasite elimination while protecting the host from disease (Maizels and Lawrence, 1991; King and Nutman, 1991). It therefore follows that the breakdown of this tolerant state and the re-activation of anti-parasite effector mechanisms is responsible for the chronic sequelae. Furthermore, the heightened responses of EN and CP patients must be qualitatively different from each other.

A dynamic model of infection has been supported by epidemiological studies charting the incidence of infection and disease through time and host age (Vannamail *et al*, 1989; McMahon *et al*, 1981). Accurate analysis is difficult because of a lack of studies with reliable assessments of morbidity. Gross elephantiasis is the least common, but most obvious, sign of obstructive lymphatic disease. In the absence of adequate physical examination the actual prevalence of chronic filarial disease may be severely underestimated. However, mathematical analysis of data from sufficiently detailed studies in Africa and India demonstrated a sequential progression from infection, microfilaraemia, and amicrofilaraemia to obstructive disease in all individuals who experience patent infection (Bundy *et al*, 1991).

The simple classification of the spectrum of infection has proved extremely useful as a basis for investigating the mechanisms of immunity and pathogenesis. The main strategy has involved contrasting the responses of individuals from each of the defined groups.

However, as methods for detecting infection and lymphatic damage have become more sensitive, reagents for dissecting human immune responses have become available, and studies have used larger population groups, it has become clear that the distinction between the groups is not as absolute as it first appeared. Furthermore, the filarial-endemic population are commonly infected with other parasites that may contribute to elevated non-specific and cross-reactive responses.

#### **1.2.4. Evidence for Immune-Mediated Resistance in Lymphatic Filariasis**

There have been two major approaches to determining whether naturally occurring protective immunity occurs in lymphatic filariasis. The first is to contrast the pattern of responses in ENs with those in microfilaraemics, who are clearly infected. Since ENs have significantly greater cellular and humoral immune responses to parasite antigens than microfilaraemics, they might be expected to recognize antigens capable of inducing protective immune responses. Such analyses have been impeded by the lack of studies in which there has been sufficiently sensitive diagnosis of the EN. However, one study of a community of 459 residents on Mauke, the Cook Islands, an area heavily endemic for subperiodic bancroftian filariasis did use strict clinical, parasitological and serological criteria to select seven "infection-free" ENs (Freedman *et al*, 1989). All seven recognised a 43-kDa larval antigen compared to age-matched microfilaraemic controls, thus providing evidence for the existence of protective immunity and a role for this antigen in its induction. However, the authors did acknowledge that the immunity putatively developed by these ENs (all of whom have had > 40 years of continuous exposure) could have evolved after clearance of a patent infection rather than reflecting a state of perfect or complete immunity.

The second approach has been to define the pattern of infection with age. Strong evidence for age-dependent development of immunity exists for another major human helminth infection, schistosomiasis (Hagan *et al*, 1991). In a *W. bancrofti* endemic area

in South India, the rate of gain of infection, based on levels of microfilaraemia, was shown to peak in the 16-20 year age-group and then decline in adulthood (Vanamail *et al*, 1989). This was proposed as evidence for acquired resistance to new infection. A study in the East Sepik province of PNG, an area of intense transmission of *W. bancrofti* infection, analyzed changes in worm burden using the PC-Ag assay over a 12 month period (Day *et al*, 1991a; Day *et al*, 1991c). The levels of PC-Ag increased significantly over this year in individuals that were <20 years of age implying a continued susceptibility to infection (Day *et al*, 1991a). These individuals possessed no anti-L3 surface antibodies (Day *et al*, 1991c). However, no change was detected in PC-Ag in subjects > 20 years of age, indicating a reduced rate of gain in infection. These adults also generated antibodies to the L3 surface. These data imply that following many years of exposure, adult residents of an endemic area acquire a degree of resistance which protects them from continuing infection with L3 while not eliminating adult worms. Resistance to super-infection or "concomitant immunity" has been described for other helminth infections (Maizels *et al*, 1993).

Evidence in support of the acquisition of natural immunity to chronic filarial infection has been provided by the cat model. It was shown initially that adult worm recoveries were much higher in cats that had been given 10 additional doses of L3 at 10 day intervals compared to those given a single infection (Denham *et al*, 1972). However, after approximately 20 challenge infections there was no further increase in the number of worms establishing, indicating that a threshold level had to be attained before generation of an immune response capable of preventing the development of the challenge infection. More recently it was shown that once the repeatedly infected cats became immune (i.e. >20 challenges) the majority of the challenge larvae were killed within the first 24h, indicating that the target of the protective immune response was the L3 (Denham *et al*, 1983). However, a significant proportion of these repeatedly infected "immune" mf- cats kill their adult worms by IgE mediated attack (Denham *et al*, 1992; Baldwin *et al*, 1993). Therefore, in the human, existing adult worms may also be

susceptible to attack even when a targetted anti-L3 response, that protects against super-infection, appears to exist.

However, the concept of concomitant immunity would still validate larval antigens as targets for vaccination since they appear to be the object of naturally occurring immune mechanisms of resistance. The mechanisms by which the immune system may eliminate larval stages remain to be defined. Natural animal hosts can be successfully vaccinated against a range of filarial worms using radiation attenuated L3 (reviewed in Denham, 1980). In the jird, three doses of *B. pahangi* larvae irradiated with 90kRad produced a 74.9% level of resistance to s.c. challenge (Chusattayanond & Denham, 1986). Oothuman *et al* (1979) demonstrated successful vaccination of cats with *B. pahangi* larvae attenuated by irradiation with 10kRad  $^{60}\text{Co}$ , a dose at which larvae develop into stunted adults. However, the mechanism by which irradiation facilitates enhanced immunity has not been characterised. One possible explanation is that irradiation inhibits normal parasite development so allowing the host immune system extended access to potentially more immunogenic/less tolerogenic antigens expressed by irradiated larval stages.

In the PNG population described by Day *et al* (1991c), no "infection-free" ENs were found. A group were negative by mf counts but positive using both the PC-Ag assay and detection of filarial-specific IgG4. Therefore, this group are displaying powerful anti-microfilarial immunity. Analysis of the responses of such individuals may uncover mechanisms capable of limiting microfilaraemia to levels that reduce the spread of infection. In a separate study of 38 individuals from the same region of PNG, sera were tested for antibody reactivity against a 25 kDa antigen that had been purified from mf extract (Kazura *et al*, 1986). Immunisation of mice with this antigen can reduce microfilaraemia. It was found that a group of 22 amicrofilaraemic individuals with low PC-Ag indices had higher anti-25 kDa antibody levels than 16 microfilaraemic residents.



### 1.2.5. Role for Antibody Isotype Responses

Initially, asymptomatic microfilaraemics were shown to have low serum anti-filarial antibody levels (Ottesen *et al*, 1982). Furthermore, stimulation of PBMCs from microfilaraemics with PWM failed to produce filarial-specific antibodies; in contrast, patients with chronic lymphatic obstruction or TPE produced large quantities of antibody (Nutman *et al*, 1987). However, PWM is a T cell-dependent B cell mitogen and direct measurement of different serum antibody isotype levels have demonstrated that microfilaraemics are not completely B cell unresponsive.

Many studies have investigated a role for different antibody isotype responses in determining the different clinical manifestations of filariasis patients. The dominant isotype of antifilarial antibody in all groups is IgG4, representing 50-95% of the total IgG response, in contrast to 4% of the total IgG in serum from non-endemics (Ottesen *et al*, 1985a; Kwan-Lim *et al*, 1990; Kurniawan *et al*, 1993). Filarial-specific IgG4 is considered to indicate the presence of active filarial infection (Kwan-Lim *et al*, 1990). It has been suggested that IgG4 antibodies are especially prominent when antigenic exposure is chronic (Aalberse *et al*, 1983). Consistent with this concept, the unusual predominance of this subclass is most pronounced in microfilaraemics, who have higher levels of filarial-specific IgG4 than pathology patients (generally mf-) (Ottesen *et al*, 1985a; Kurniawan *et al*, 1993; Yazdanbakhsh *et al*, 1993). However, a sub-population of *W. bancrofti* microfilaraemics have been described with low IgG4, but high levels of mf and PC-Ag (Marley *et al*, 1995).

Serum IgE levels (antigen-specific and polyclonal) are also elevated in patients with lymphatic filariasis (Hussain *et al*, 1981; Hussain and Ottesen, 1986; Hitch *et al*, 1989; Kurniawan *et al*, 1993). This is a common feature of many helminth infections and has led to the hypothesis that IgE evolved to selectively fight extracellular parasites

(Finkelman *et al*, 1991). IgE mediates immediate hypersensitivity reactions. Antigen cross-links specific IgE bound to Fcε receptors on the surface of mast cells and basophils so inducing degranulation. Cats, repeatedly infected with *B. pahangi*, that kill their adult worms generate good filarial-specific IgE responses (Baldwin *et al*, 1993). In contrast, those cats that tolerate their parasites do not make IgE responses even after treatment with macrofilaricidal anthelmintics. This suggests that IgE production in cats is responsible for, rather than a consequence of, the death of adult worms in the lymphatics. In humans infected with schistosomiasis, levels of parasite-specific IgE increase over time, coincident with the emergence of age-related immunity to the invasive schistosomula (Hagan *et al*, 1991). In brugian and bancroftian filariasis, specific IgE levels are the lowest in the most heavily parasitized group, the microfilaraemics, and highest in filariasis patients with CP (Hussain *et al*, 1981; Kurniawan *et al*, 1993; Yazdanbakhsh *et al*, 1993), although there is also a minor subset of elephantiasis patients with brugian filariasis who have low specific IgE levels (Yazdanbakhsh *et al*, 1993). In trichuriasis, IgE-mediated hypersensitivity is linked with pathology rather than resistance (Cooper *et al*, 1991).

The relationship between IgG4 and IgE is particularly intriguing. Both isotypes can be directed against the same antigenic determinants (Ottesen *et al*, 1981) and both are known to be promoted by IL-4 and inhibited by IFN-γ (King *et al*, 1990; King and Nutman, 1993). It has been suggested that IgG4 may serve to modulate IgE mediated allergic reactivity by acting as a "blocking antibody" (Ottesen *et al*, 1981; Hussain *et al*, 1992). Filariasis patients, with the exception of individuals with TPE, do not show any signs of allergy, despite specific IgE levels that are as high or higher than those reported in allergic individuals with ragweed or grass allergies (Hussain *et al*, 1981; Gleich *et al*, 1977). TPE is a distinctive condition. Specific IgE in TPE patients is significantly greater than levels of total IgE in atopic patients and levels of specific IgE in the other filariasis groups (Ottesen and Nutman, 1992).

While IgG4 and IgE are often induced in parallel, differential regulation is also apparent when responses are examined quantitatively. The majority of asymptomatic microfilaraemics have extremely high levels of specific IgG4 and low specific IgE responses relative to amicrofilaraemic elephantiasis patients, who tend to display the reverse relationship (Kurniawan *et al*, 1993). In addition, in schistosomiasis IgG4 levels decrease over time relative to increasing IgE (Hagan *et al*, 1991). Similarly, in filariasis IgG4 levels peak in childhood (Hitch *et al*, 1991), whereas IgE levels continue to increase with age (Hitch *et al*, 1989). Differential expression of IgG4 and IgE in human B cells has also been observed experimentally (Kimata *et al*, 1991).

The expression of IgG1, IgG2 and IgG3 in filariasis is in marked contrast to that of IgG4. Elephantiasis patients have significantly higher levels of IgG1, IgG2 and IgG3 than asymptomatic microfilaraemics, who make very little of these subclasses (Ottesen *et al*, 1985a; Hussain *et al*, 1987; Yazdanbakhsh *et al*, 1993). It has been suggested that IgG1, 2 and 3 antibodies mediate inflammatory reactions in response to dying adult worms in the lymphatics (Yazdanbakhsh *et al*, 1993). All three isotypes exhibit complement fixing activity. In addition, IgG1 and IgG3 antibodies have been implicated in well-characterised tissue damaging type III hypersensitivity reactions, that have been compared with the oedematous lesions in filariasis (Hussain *et al*, 1987). Circulating immune complexes and low concentrations of C3 (due to utilisation?) have been observed in the serum of patients with elephantiasis (Ottesen, 1980; Ottesen *et al*, 1982). In human onchocerciasis, specific IgG3 antibodies are apparent in individuals with the chronic hyperreactive onchodermatitis condition called Sowda (Cabrera *et al*, 1988). However, a protective role for IgG3 in individuals who are putatively immune to infection with onchocerciasis has also been suggested (Boyer *et al*, 1991). A study of a Haitian pediatric population (6-10 years old) showed that amicrofilaraemic children displayed elevated levels of IgG2 and IgG3 in comparison to microfilaraemic children infected with *W. bancrofti* (Hitch *et al*, 1991).

In the face of apparently conflicting data, the exact contribution of IgE-mediated and IgG1, 2 and 3-mediated reactions to the pathology of filariasis remains to be established. It is interesting to note that IgG4, the predominant isotype in asymptomatic carriers, is also unable to fix complement and therefore may serve to dampen pathogenic processes on two levels: by blocking Type III hypersensitivity reactions as well as IgE mediated Type I reactions.

#### **1.2.6. Role for T Cell Responses**

Most asymptomatic mf+ are unable to generate antigen-specific T cell proliferative responses *in vitro*, in contrast to the vigorous responses of T cells from endemic normals and elephantiasis patients (Ottesen *et al*, 1977; Piessens *et al*, 1980; Lammie *et al*, 1988; Hitch *et al*, 1991; Yazdanbakhsh *et al*, 1993). The T cell blastogenic response, by its dependency on IL-2 (produced by Th1 but not Th2 cells), is recognised as a measure of Th1 reactivity. When the cytokine profiles of the various groups were analyzed independently it was found that peripheral T cells from microfilaraemics produced significantly less antigen-specific IL-2 and IFN- $\gamma$  than T cells from endemic normals or elephantiasis patients (Nutman *et al*, 1987a; King *et al*, 1993; Maizels *et al*, 1995). In contrast, T cells from microfilaraemics, as well as ENs and elephantiasis patients, secrete significant levels of IL-4 when stimulated with antigen (King *et al*, 1993) or PHA (Yazdanbakhsh *et al*, 1993a). This is consistent with a specific Th1 defect in the cellular response of microfilaraemics, leaving Th2 activity (IL-4 and IgG4 production) enhanced in their absence.

The fact that the level of IL-4 in all infected individuals is elevated in comparison to non-endemic controls suggests that there is a Th2 biased response to active infection (Yazdanbakhsh *et al*, 1993a). Consistent with this opinion, it has been shown that CD4 cells secreting IL-4 and IL-5 are selectively expanded in lymphatic filarial infection and may result in the characteristic elevation in IgG4 and IgE isotypes and prominent eosinophilia (Mahanty *et al*, 1993). Since IL-4 production by PBMCs elevated in all

groups, the precise role of Th2 cells in the development of immunity or disease is unclear.

Originally, in studies with relatively small sample sizes, the Th1 defect was found only in clinically asymptomatic individuals with circulating mf and therefore it was proposed that adult worms and/or mf are responsible for the induction of Th1 tolerance. Furthermore, the development of pathology is generally accompanied by the loss of circulating mf, and expression of proliferative T cell responses and IFN- $\gamma$  production (King *et al*, 1993; Maizels *et al*, 1995). In addition, treatment of microfilaraemics with DEC results in the restoration of antigen-specific proliferation and IFN- $\gamma$  release (Lammie *et al*, 1988a; Sartono *et al*, 1995). However, when much larger population groups are examined a significant degree of heterogeneity was revealed within the clinical categories (Ottesen, 1989). In one such study, where T cell responses and Ab isotypes were measured in the same individuals, 37% of elephantiasis patients, 32% of asymptomatic and 58% of symptomatic amicrofilaraemics were unable to proliferate in response to filarial antigens but displayed high IgG4 levels (Yazdanbashskh *et al*, 1993). Therefore, it appears that the presence of active infection may result in Th1 hyporesponsiveness, irrespective of clinical category. One implication of this is that life cycle stages, other than mf, can down-regulate responses. In fact, the reduction in antigen-driven IFN- $\gamma$  production is related to the asymptomatic state-irrespective of mf status (Maizels *et al*, 1995).

Induction of Th1 unresponsiveness may not offer complete protection from disease since asymptomatic mf+ individuals can have sub-clinical abnormalities in renal and lymphatic function (Ottesen and Ramachandran, 1995; Freedman *et al*, 1995). In contrast, not all pathology patients exhibit elevated Th1 cell responses (Maizels *et al*, 1995). Furthermore, DEC treatment elevates T cell responses and IFN- $\gamma$  production in unresponsive individuals from all clinical categories (Sartono *et al*, 1995), yet alleviates some symptoms of chronic lymphatic disease (Partono *et al*, 1989) which would argue

against these factors being components in the development of elephantiasis. These data indicate that multiple immunological mechanisms may account for antigen-specific unresponsiveness (1.2.7) and development of disease (1.2.8).

### **1.2.7. Possible Mechanisms of T Cell Unresponsiveness**

Various mechanisms have been suggested to explain the parasite-specific Th1 hyporesponsiveness observed in lymphatic filariasis:

- tolerance
- clonal deletion
- suppression

#### **(1) Tolerance**

Th1 cell tolerance or anergy can result from inappropriate antigen presentation. T cells require signalling through the Ag receptor and co-stimulatory pathways to become fully activated and initiate endogenous IL-2 production (Schwartz, 1990). In the absence of this "second signal" T cells can enter a state of unresponsiveness that is not reversed even when antigen is then "appropriately" presented (Jenkins and Schwartz, 1987). Th1 clones are more susceptible to tolerance induction than Th2 clones (Williams *et al*, 1990; Gajewski *et al*, 1994). A mechanism of clonal anergy is supported by the reduced precursor frequency of parasite-specific T and B lymphocytes in microfilaraemics versus elephantiasis patients (King *et al*, 1992).

*In vitro* hyporesponsiveness in onchocerciasis can be restored by the addition of exogenous IL-2, indicating that a lack of IL-2 production may be responsible for the proliferative defect in filariasis (Gallin *et al*, 1988). In one study the addition of rIL-2 reversed antigen unresponsive in cells from 12-28% of individuals with brugian filariasis (Sartono *et al*, 1995a). That particular study also tested a variety of antibodies

to known costimulatory molecules CD2, CD28, CD26, CD27 and pharmacological mediators. In general, these treatments had no effect on antigen unresponsiveness in samples tested. However, antigen unresponsive cells from some individuals did respond to treatment with PMA and indomethacin. When these data were combined with the IL-2 treatment data, it was found that unresponsiveness is more easily reversible in elephantiasis patients (50%, 9/18) than in microfilaraemics (12.5%, 4/32) or ENs (20%, 3/15). This is consistent with a greater enhancement in antigen responsiveness in elephantiasis patients versus microfilaraemics following DEC treatment (Sartono *et al*, 1995), and thus a more extreme state of anergy in microfilaraemics than the other two groups.

Chronic antigen stimulation/exhaustion could be another mechanism of tolerance induction, consistent with the ability of DEC treatment to partially restore proliferation and IFN- $\gamma$  production, perhaps by reducing the parasite load. In a murine model of tolerance to H-Y antigen, continual exposure of T cells to antigen results in T cell anergy (Ramsdell and Fowlkes, 1992). CD27 is upregulated on activation and is shed from the T cell surface into body fluids (Rubin *et al*, 1985). Upon repeated stimulation T cells remain CD27<sup>-</sup>, suggesting a link between chronic immune activation and an increase in CD27<sup>-</sup> T cells. In one study, it was found that CD4<sup>+</sup>CD27<sup>-</sup> T cells were significantly expanded and sCD27 levels raised in microfilaraemics and in elephantiasis patients compared with ENs (Yazdanbakhsh *et al*, 1993a). The evidence for chronic T cell stimulation in microfilaraemics is interesting in relation to the inability of these cells to proliferate *in vitro*. When anergic cells, from the H-Y model system, were maintained in culture in the absence of antigen but in the presence of IL-7 (a T cell trophic factor) responsiveness was restored (Ramsdell and Fowlkes, 1992). However, similar treatment of cells from brugian filariasis patients failed to reverse their unresponsiveness (Sartono *et al*, 1995a).

It was speculated that the difficulty experienced in reversing antigen unresponsiveness in brugian filariasis patients could reflect a more profound level of anergy than in other systems (Sartono *et al*, 1995a). However, the cells from filariasis patients had been cryopreserved, a process that may have affected critical biochemical pathways that mediate recovery from the anergic state.

## **(2) Clonal deletion**

Children born to mothers infected with *W. bancrofti* were observed to be more susceptible to filarial infection than those born to uninfected mothers (Lammie *et al*, 1991). In that study, of 1034 individuals in 237 households in Haiti, it was shown that children born of a microfilaraemic mother were 3.5 times more likely to be microfilaraemic than children whose mother was amicrofilaraemic. This is unlikely to be due to familial differences in exposure since the infection status of offspring was not related to paternal status.

It was hypothesised that the increased susceptibility of children born to infected mothers is due to the deletion of antigen-specific lymphocytes *in utero* by exposure to filariae or filarial antigens during thymic development. The presence of anti-filarial IgM and IgE (isotypes that are unable to cross the placenta) in cord blood (Weil *et al*, 1983) supports the concept of *in utero* exposure to infection.

In a more recent study, Steel *et al* (1994) showed that individuals from Mauke (Cook Islands), born to *W. bancrofti* microfilaraemic mothers, in contrast to those born to amicrofilaraemic mothers, were unresponsive to parasite antigen *in vitro* nearly twenty years later. The long-term unresponsiveness exhibited by the offspring of infected mothers extended to both Th1 and Th2 type cytokines and is therefore different from that of chronically infected individuals, which is a specific Th1 defect, consistent with a further more permanent mechanism of tolerance.



Clonal deletion of antigen reactive T cells would also explain why infection in the indigenous population and their descendants usually results in non-responsive asymptomatic microfilaraemia whereas expatriates (e.g. military personnel) and transmigrants (with identical ethnic background to the endemic population) tend to develop hyper-reactive lymphatic inflammation more often and quicker (Partono *et al*, 1978). Consistent with these observations, male progeny of infected female jirds exhibited reduced lymphatic lesion severity compared to similarly infected progeny of uninfected female jirds (Klei *et al*, 1986).

However, it cannot be ruled out that antigen-specific T cell clones are rendered anergic rather than deleted by *in utero* exposure to filariae. The ability to reverse antigen-specific unresponsiveness by DEC treatment suggests that reversible mechanisms of tolerance such as anergy are generally operative and/or deletion is incomplete.

### **(3) Suppression**

Adherent cells and suppressor T lymphocytes have both been implicated as mediators of parasite-specific unresponsiveness in filariasis (Piessens *et al*, 1980; Piessens *et al*, 1982). However, a more recent study could find no evidence that removal of adherent cells or CD8<sup>+</sup> T cells had any effect on antigen unresponsiveness in microfilaraemics infected with *W. bancrofti* (Nutman *et al*, 1987; Nutman *et al*, 1987a). Serum from microfilaraemics has been shown to selectively suppress antigen-induced proliferation of cells from amicrofilaraemics (Piessens *et al*, 1980), suggesting that suppression by filarial antigens may occur.

Since Th2 responses are elevated in filarial infection, it is possible that the secretion of high levels of Th2-dependent cytokines could contribute to suppressing Th1 cell activity. It has been shown that treatment of cells from *W. bancrofti* microfilaraemics with anti-IL-10 can significantly enhance antigen-specific proliferation (220-1300%) (King *et al*, 1993). Neutralisation of TGF- $\beta$ , another cytokine that can suppress T cell

proliferation, also increased antigen-driven proliferation. However, treatment with anti-IL-10 or anti-IL-4 had no effect on the proliferation of cells from antigen-nonresponsive *B. malayi* infected individuals in each of the clinical categories (Sartono *et al*, 1995a). It is possible that induction of unresponsiveness in *W. bancrofti* infection differs from that of brugian filariasis. In some individuals the non-responsive state induced *in vivo* may be too profound to be recovered by short-term *in vitro* treatment. In this context, IL-10 can induce T cell anergy by downregulating APC function (Villanueva *et al*, 1993).

T cell unresponsiveness to PPD (a Th1 inducing antigen) observed in some filarial patients can be restored after DEC treatment, suggesting that responses to non-filarial antigens may be affected by the generalized skewing of the cytokine response in the Th2 direction (Soboslay *et al*, 1994; Sartono *et al*, 1995). However, despite the Th2 bias in lymphatic filariasis, the cytokine profile in response to PPD in *B. malayi* infection remains exclusively Th1 (Sartono *et al*, 1996). Recent studies in PNG (C. King pers. comm.) suggest that immune responses are generally depressed coincident with high levels of infection.

### **1.2.8. Determinants of Pathology**

CP is now considered to have at least three components, each of which may or may not contribute to CP in every patient:

- immunological
- parasitological
- microbiological

## **(1) Immunological**

CP patients are generally mf- and hyper-responsive in that they can display heightened T cell proliferative responses, elevated levels of IL-4 and IFN- $\gamma$ , and high levels of IgE, IgG1, 2 and 3 to filarial antigens (Maizels *et al*, 1995). Therefore, inflammatory T cell directed responses leading to/or as a consequence of parasite death may be the cause of the severe lymphatic lesions. The low levels of IgG4 in these patients is associated with parasite death suggesting that disease has out-lived the filarial infection.

However, a significant proportion of CP patients are mf+, T cell hyporesponsive and have high ratio of IgG4/IgE (Maizels *et al*, 1995). In these individuals direct parasitological factors (see below) may predominate. In addition, a proportion of ENs also have high IgE levels and T cell responses. It has been suggested that these individuals do not develop pathology because incoming larvae are killed before they reach the lymphatics. It is interesting to note that high levels of anti-parasite IgG1, 2 and 3 is exclusive to CP patients and that these sub-classes are elevated irrespective of T cell and IgE responses. Therefore, IgG1, 2 and 3 induced by dying worms (killed by whatever mechanism/s) may mediate hypersensitivity reactions resulting in elephantiasis.

In the light of recent diagnostic advances, it is now recognised that essentially all infected individuals possess either the overt symptoms of pathology or subclinical abnormalities (Ottesen and Ramachandran, 1995). Progression from the microfilaraemic to an amicrofilaraemic state, lack of *in utero* exposure, and length of exposure (elephantiasis patients are significantly older than asymptomatic microfilaraemics) are all risk factors for the development of pathology (Ottesen, 1992). However, the fact that a minority of the infected population eventually develop obstructive disease suggests that genetic factors e.g. HLA genes may also play a controlling role. One study found a negative association between the usage of a particular HLA-DQ epitope and the development of pathology in brugian filariasis patients in Indonesia (Maizels *et al*,

1995). Similarly, in onchocerciasis specific HLA-DR epitopes have been correlated with Sowda (Brattig *et al*, 1986) or freedom from disease (Meyer *et al*, 1994).

## **(2) Parasitological**

The parasitological element reflects the ability of adult worms to directly induce lymphatic dilation and hence lymphadenitis by reducing lymphatic function (Vincent *et al*, 1984). Although this data has come from experiments performed in a mouse model, a similar phenomenon is considered to be operating in the lymphatic system of infected humans. Human lymphatic endothelial cell function can be depressed by filarial parasite secretory products (Kaiser *et al*, 1990). In addition, the adults living in the lymphatics are constantly motile and may cause direct structural damage (Case *et al*, 1992).

## **(3) Microbiological**

The significance of secondary opportunistic infections in the pathogenesis of filarial disease has only recently come to light. Both bacterial and fungal infections have been implicated in exploiting the lymphatic damage caused by the filarial infection itself. Improvements in local hygiene plus treatment with antibiotics and anti-fungal agents can prevent ADL and reverse the early occurrence of lymphodema (Ottesen and Ramachandran, 1995).

### 1.3. Animal Models of Lymphatic Filariasis

Both *B. malayi* and *B. pahangi* have been used as animal models of filariasis. The only non-human host in which *W. bancrofti* is known to develop is the silver leaf monkey, *Prebytis cristatus*, and no laboratory models have yet been developed.

#### 1.3.1. The Jird

The Mongolian jird, *Meriones unguiculatus*, is a permissive host for both *B. pahangi* and the sub-periodic form of *B. malayi* (Ash and Riley, 1970; 1970a). Following s.c. inoculation with L3, most adults localised to the regional lymphatics from where they release mf into the bloodstream (Ah and Thompson, 1973). The jird model has played a vital role in addressing some basic aspects of immunity, immunosuppression and pathology in filariasis. An early acute response to the parasite is followed by the development of a state of chronic microfilaraemia and depressed cellular responses that may persist for several years. Jirds can display high levels of resistance to infection following immunisation with irradiated larval vaccines (Chusattayanond & Denham, 1986). Ah & Thompson (1973) first described the lymphatic pathology associated with *B. pahangi* infections in the jird. The intra-lymphatic granulomatous lesions in jirds are histologically similar to those described in human filariasis patients (Vincent *et al*, 1980). Sensitisation with parasite antigens prior to infection increased lymphatic lesion severity, supporting a role for heightened anti-parasite immune responses in lymphatic lesion pathogenesis (Klei *et al*, 1982). The early inflammatory response in the jird, with the associated granuloma formation, is downregulated prior to/or at the time of adult parasite maturation and mf release (Rao *et al*, 1996).

The dynamics of the pathological responses in jirds is clearly the opposite to that of many CP patients who develop strong anti-parasite responses but are mf-. However, the response of chronically infected jirds may relate to the category of CP patients that are T cell hyporesponsive and mf+. Multiple infections do not significantly increase

granuloma numbers (Klei *et al*, 1990) and existing chronic intraperitoneal infections reduce the development of these lesions during a subsequent lymphatic infection (Klei *et al*, 1987), indicating that existing infection in the susceptible jird downregulates inflammatory anti-parasite responses, as has been suggested to occur in the asymptomatic microfilaraemic human. Furthermore, the finding that Th1 hyporesponsiveness can occur in actively infected humans, irrespective of clinical category, are in agreement with studies in the jird model that have shown that the maintenance of depressed cellular responses in infected animals is not dependent on microfilaraemia (Bosshardt *et al*, 1995), although the levels of mf or adults worms do appear to have some effect on the level of this downregulation (Klei *et al*, 1990; Bosshardt *et al*, 1995). Finally, i.p. inoculation with L3 yields large quantities of adults worms and mf contained within the peritoneal cavity and so cyclical passage through the jird has become the most common method for maintaining laboratory stocks of the parasite.

### **1.3.2. The Cat**

The cat, a natural host of *B. pahangi*, has been promoted as a model for studying the relationships between infection, immunity, and disease dynamics in LF (Grenfell *et al*, 1991). The life cycle, longevity of infection, and the clinical sequelae closely resemble those of *Brugia* and *Wuchereria* in humans (Denham and Fletcher, 1987). Cats repeatedly inoculated with L3 can be classified into five groups based on a combination of detection of microfilaraemia (mf+), circulating antigen (CAg, indicative of the presence of living adults), and the presence of adult worms in the lymphatics at autopsy after 3 years (Denham *et al*, 1992). This range of outcomes reflects the complexity of the human infection and reveals some of the problems associated with studying the human disease. For example, four out of the five groups could represent the human endemic normal i.e. amicrofilaraemic (mf-), yet clearly some of the cats have been patently infected while others harbour occult infections. Studies in the cat also highlight the heterogeneity that exists in the other clinical groups. After 20 doses of L3, cats

display resistance to re-infection, providing strong evidence for the development of natural immunity to chronic filarial infection in a susceptible host. However, repeatedly infected cats, both mf+ and mf-, can also display pathological changes .

### 1.3.3. The Mouse

At present the mouse is the only experimental host in which detailed and informative immunological studies can be carried out. Intact mice are resistant to infection, although single parasite stages can be implanted into the peritoneum and survive for a limited period. Following i.p. infection with *B. pahangi* L3, 10-40% larval recoveries could be obtained 28 days later in CBA/Ca, BALB/c and asplenic (Dh/+) mice suggesting a limited susceptibility to *B. pahangi* infection in some strains (Howells *et al*, 1983). In a separate study, a number of mouse strains including BALB/c mice were susceptible to *B. malayi* larval development, through the L3 to L4 moult, for 2 weeks after i.p. inoculation (Hayashi *et al*, 1989). Adult worms can be implanted i.p. into normal mice and survive for 90 days, generating a high level of microfilaraemia (Suswillo *et al*, 1980). Similarly, isolated mf can survive for 28 days in the peritoneal cavity after i.p. inoculation (Rajasekariah *et al*, 1988) and following i.v. infection have been found in the blood from 65 days (Fanning and Kazura, 1983) to 120 days later (Grove *et al*, 1979).

In contrast, infection of congenitally athymic (nude) (Vincent *et al*, 1980) or thymectomised mice (Suswillo *et al*, 1980) with *B. pahangi* L3 results in a fully patent infection. The susceptibility of the nude mice is equivalent to that of the jird. An average of 15% of larvae inoculated s.c. into nudes can be recovered as adult worms and nearly 100% of infected nudes become microfilaraemic (Vincent *et al*, 1980). Furthermore, the development of the parasite occurs normally, with a proportion of the larvae maturing within the lymphatics. As in the natural host (the cat), the L3 to L4 moult occurred at 7-10 days. Likewise, the final moult to the adult stage occurred at about 24 days for males and 33 days for female worms (Vincent *et al*, 1982). Not

surprisingly, SCID mice (which lack functional T and B lymphocytes) are also permissive to infection (Nelson *et al*, 1991).

The use of immunodeficient mice have been instrumental in dissecting the mechanisms of murine resistance to *Brugia*. A T cell-mediated mechanism of resistance operates against the immature larvae since athymic and SCID mice, but not normal mice, are susceptible to *Brugia* infection (Suswillo *et al*, 1980; Vincent *et al*, 1980; Nelson *et al*, 1991). Furthermore, athymic mice can be made resistant to L3 by thymic grafting, injection of thymocytes or cyclosporin A resistant Thy1<sup>+</sup> primed splenocytes (Vickery *et al*, 1983; Vickery and Nayar, 1987). Reconstituted nude mice eliminate the infection in the early larval stages. However, transfer of B cells or immune serum to the nude recipients had no effect on parasite clearance. Similarly, the transfer of splenocytes but not serum from vaccinated BALB/c mice enhances the clearance of larvae in the recipients 2 weeks after i.p. challenge (Hayashi *et al*, 1984).

Using defined mutant or KO mice, attempts have been made recently to determine more precisely the T cell population that orchestrates resistance.  $\beta$ 2mKO mice do not express Class I or Class I related MHC molecules and therefore do not permit development of conventional Class-I restricted CD8<sup>+</sup> T cells. CD4 KO mice have a null mutation in the gene encoding CD4 and, therefore, have impaired development of CD4<sup>+</sup> helper T cells. However, both  $\beta$ 2mKO (Rajan *et al*, 1992) and CD4 KO (Rajan *et al*, 1994) mice are completely resistant to *B. malayi*. No adult worms could be recovered at 12 weeks post L3 infection. These experiments appear to rule out the involvement CD8 and CD4 T cells in killing L3. However, it should be noted that 12 weeks is very late on in infection to assess a role for these cells in larval killing which, in normal mice, occurs mainly within the first two weeks (Carlow and Phillip, 1987). Therefore, it is possible that, in the absence of CD8 or CD4 T cells, larval killing was delayed or prevented and that a later stage was the target. In addition, in neither study was the survival of infective larvae assessed in equivalent wild-type animals. Strain differences in murine



susceptibility have been reported (Howells *et al*, 1983). Furthermore, CD4<sup>+</sup>CD8<sup>-</sup> T cells with helper activity have been shown to mediate protection against *Leishmania major* in CD4 KO mice (Locksley *et al*, 1993). Compensatory T cell populations in CD4 and CD8 KO mice may be similarly effective against *Brugia*. Therefore, evidence for or against an obligate requirement for CD4 and CD8 T cells in murine resistance to brugian parasites remains to be convincingly demonstrated. Adults implanted i.p. survive longer in athymic mice than in their thymic counterparts (Suswillo *et al*, 1980) indicating that adults worm are killed by T cell-dependent mechanisms. Consistent with this, adults that have become established in these athymic mice can be killed upon reconstitution with primed splenocytes (Vickery *et al*, 1991).

The mouse has also been indispensable in the study of antibody-dependent effector mechanisms, that appears to be the predominant mechanism of anti-mf immunity. Treatment of mice with anti-mf MAbs enhances clearance of mf from the bloodstream (Aggarwal *et al*, 1985). In addition, CBA/N mice, that have a defect in B cells resulting in reduced IgM responses, remain patent after i.v. re-injection of mf, unlike normal CBA/H mice that clear mf rapidly and remain mf- after re-injection (Thompson *et al*, 1981). Furthermore, transfer of sera from immune Swiss Webster to naive recipients mice results in a 75-100% protection against challenge with mf (Kazura and Davis, 1982). The predominant antibody isotype in the immune sera is IgG2a, although a direct role for this isotype has not been demonstrated.

The mechanisms by which cellular and antibody responses may co-ordinate to kill filarial parasites is very poorly understood. ADCC mediated killing of infective larvae and mf *in vitro* by eosinophils, macrophages and neutrophils has been reported (Chandrashekar *et al*, 1985). Interestingly, *Brugia* parasites contains surface and secreted molecules with anti-oxidant activity that may function to repair or protect against immune mediated oxidative damage (Tang *et al*, 1994; Tang *et al*, 1996).

Histological examination of granulomas, from infected jirds and mice, containing dead and dying worms has been used to identify cells that may be directly involved in worm killing. In jirds, Yates and Higashi (1986) reported structural damage to the L3 cuticle associated with the presence of eosinophils. Eosinophil degranulation and entry into the damaged cuticle were observed. In immune-reconstituted nude mice large granular macrophages, giant cells and eosinophils were the predominant cell types in the granulomas associated with killing of adult worms (Vickery *et al*, 1991).

Nude mice chronically infected with *B. pahangi* develop persistent lymphodema in affected limbs and progressive lymphangiectasis resulting in massive lymphatic dilation (Vincent *et al*, 1984). Similar changes were observed in infected SCID mice (Nelson *et al*, 1991). Surgical removal of the worms from nude mice reversed the condition and its development was not correlated with microfilaraemia. Despite the term "elephantoid" nude mouse, the chronically-infected nude mouse does not display many of the characteristics of filarial elephantiasis. The condition is reversible and the lymph thrombi formed are non-obstructive. In contrast, immune-reconstitution of chronically infected nude mice with primed splenocytes results in obstructive lesions within dilated lymphatics (Vickery *et al*, 1991). The development of these lesions was associated with the destruction of adult worms and mf within granulomas composed of eosinophils, giant cells and large granular macrophages. Transfer of defined cell populations may provide further information on the different levels and mechanisms of pathology.

A further interesting observation from these studies was that *B. pahangi* L3 were strongly pathogenic to the lymphatic vessels of the immunologically normal thymic counterparts. Severe inflammation developed rapidly and including areas of necrotising lymphangitis and involving tissues other than the lymphatics. These findings contrast with studies on susceptible hosts that concluded that "early filarial larvae are the least pathogenic stages of *Brugia*" (Denham and McGreevy, 1977) and that stage-specific L3

antigens could be used in vaccines without fear of provoking disease (Maizels and Lawrence, 1991).

## 1.4. Immunoregulation I: Th Cell Development and Function

### 1.4.1. The Concept of Th1 and Th2 Cells

The concept of Th1 and Th2 cells was based originally on the ability of long-term murine CD4<sup>+</sup> T cell clones to secrete either IL-2, IFN- $\gamma$ , TNF- $\beta$  and LT (Th1 clones) or IL-4, IL-5, IL-6, IL-10 and IL-13 (Th2 clones), although there are a few cytokines that are produced by both (e.g. IL-3 and GM-CSF) (Mosmann *et al*, 1986; Street and Mosmann, 1991; Sher and Coffman, 1992; Seder and Paul, 1994). In the following years the *in vivo* existence and biological significance of Th cell subsets resembling Th cell clones has been demonstrated convincingly.

Through their distinctive pattern of cytokine production, Th1 cells mediate cell-mediated immune responses (macrophage activation, IgG2a production, and DTH) that are important for the eradication of intracellular organisms, while Th2 cells provide optimal help for B cells (humoral responses) and for allergic responses by activation of mast cells and eosinophils (Mosmann and Coffman, 1989; Reiner and Locksley, 1993; Seder and Paul, 1994). The ability of IFN- $\gamma$  to inhibit Th2 activity (Gajewski and Fitch, 1988) and IL-4 and IL-10 to inhibit Th1 responses (Powrie *et al*, 1993) means that once selected, Th cell responses can become rapidly polarized. This may in part explain the mutually exclusive nature of cell mediated and humoral responses (Parish, 1972).

Th cells producing cytokines typical of both Th1 and Th2 clones have been observed in both murine and human systems (Umetsu *et al*, 1988; Firestein *et al*, 1989; Elson *et al*, 1995; Openshaw *et al*, 1995). These have been called Th0 cells and may be an intermediate stage towards the polarized Th1-type and Th2-type phenotypes (Kamogawa *et al*, 1993). Alternatively, Th0 cells may represent a separate, stably differentiated population that develop when conditions are less polarizing. It is possible

that Th0 cells function where the correct balance of cell-mediated and humoral immunity can eliminate the antigenic stimulus with a minimum of immunopathology.

*In vivo*, Th cells are thought to exist as either naive, effector or memory cell-types (Swain *et al*, 1990). Upon primary stimulation naive CD4<sup>+</sup> T cells (precursor, pTh) produce mainly IL-2 and dominate in the thymus and lymphoid organs of young, immunologically naive mice. Following re-stimulation pTh cells differentiate into effector Th1, Th2 or Th0 cells that are relatively short-lived. Both pTh and effector Th cells disappear following adult thymectomy. However, the resting CD4<sup>+</sup> T cells that persist rapidly produce IL-2, IL-4 and/or IFN- $\gamma$  after re-stimulation and are known as memory Th cells.

Initially, polarized Th populations were difficult to observe in humans. However, it is now clear that Th1-type and Th2-type CD4<sup>+</sup> T cells can be isolated from PBMCs during chronic infectious diseases and allergic conditions, consistent with the original discovery of murine Th1 and Th2 cells as clones following chronic *in vitro* antigen stimulation. Most allergen specific T cell clones from atopic donors (Wierenga *et al*, 1990) and T cell clones specific for *Toxocara canis* excretory/secretory antigens exhibit a Th2 profile (Del Prete 1991). In contrast, the majority of T cell clones specific for PPD of *Mycobacterium tuberculosis* show a Th1 profile (Del Prete *et al*, 1991).

Ig isotypes are considered to be *in vivo* indicators of particular Th cell activity. IL-4 and IFN- $\gamma$ /IL-12 appear to antagonize the effects of each other on B lymphocytes. In the mouse, IgG2a, IgG3 and IgG2b are inhibited by IL-4 and up-regulated by IFN- $\gamma$  and IL-12 (Snapper and Paul, 1987; Germann *et al*, 1995), while the switch to IgG1 and IgE isotypes is enhanced by IL-4 (Finkelman *et al*, 1990). A mixture of these isotypes is associated with a Th0 type response (Finkelman *et al*, 1990).

The concept of Th1 and Th2 cells not only explains the mechanisms of different protective responses, but also provides the pathogenic basis for several immunological diseases. Strong evidence suggests that unrestrained Th2 responses are responsible for the allergic responses to environmental antigens, whereas Th1 dominated responses are involved in other hypersensitivity reactions such as contact dermatitis as well as organ-specific autoimmune diseases (Druet *et al*, 1996).

Th polarized responses are also dependent on the production of Th1 and Th2-associated cytokines made by non-CD4<sup>+</sup> T cells. Mast cells, basophils and eosinophils can produce IL-4, IL-5, IL-6 and IL-13 and CD8<sup>+</sup> cells and  $\gamma\delta$  T cells can produce both Th1 and Th2 type cytokines (Paul *et al*, 1993; Croft *et al*, 1994; Ferrick *et al*, 1995). In addition, IL-12, produced by phagocytic cells in response to bacteria, bacterial products and intracellular parasites, induces optimal IFN- $\gamma$  production from NK and T cells (D'Andrea *et al*, 1992; Scott, 1993; Hsieh *et al*, 1993; Gazzinelli *et al*, 1993). These cell types are thought to provide a functional bridge between the activation of immediate/innate immune mechanisms and the induction of an adaptive Th response.

#### **1.4.2. Are There Cell-Surface Markers of Th1 and Th2 Cells?**

In view of the complexity in defining Th subsets based on the cytokines they secrete, the search has been on for cell-surface markers that would distinguish fully-differentiated, effector Th1 from Th2 cells. A variety of cell surface molecules have been proposed to be differentially expressed with varying and sometimes conflicting results (reviewed in Almerigogna *et al*, 1996).

For example, Powrie *et al* (1994) demonstrated that primed Th1 cells were present in *L. major*-infected BALB/c mice and could be distinguished from antigen-reactive Th2 cells based on the level of expression of CD45RB; the CD4<sup>+</sup>CD45RB<sup>hi</sup> population contained cells that secreted antigen-specific IFN- $\gamma$  while the CD4<sup>+</sup>CD45RB<sup>lo</sup>

population contained cells that secreted antigen-specific IL-4. These sub-populations were stable upon transfer to recipient SCID mice. However, treatment of the recipient SCID mice with anti-IL4 at the time of transfer of CD4<sup>+</sup>CD45RB<sup>lo</sup> cells resulted in a Th1 response, indicating that the CD45RB<sup>lo</sup> population is not exclusively Th2. Consistent with this result, the recall response to KLH in mice infected with *Bordetella pertussis* was shown to be contained within the CD45RB<sup>lo</sup> population, that secreted both Th1 and Th2 cytokines (Bradley *et al*, 1992).

Similarly, while the expression of CD30 antigen on human CD4<sup>+</sup> T cells has been linked to the production of Th2 cytokines (Del Prete *et al*, 1995), other studies have shown that the CD30<sup>+</sup> population can also contain Th0 and Th1 cell types (Hamann *et al*, 1996, Alzona *et al*, 1995). The general conclusion from these studies is that Th1, Th2 and Th0 cells share the expression of a number of cell surface molecules and that the reliable and constitutive expression of a distinct marker(s) has yet to be demonstrated.

However, these reagents have allowed a distinction to be made between naive, precursor Th cells and effector Th1, Th2 or Th0 cells. Naive Th cells are small and dense and express the CD44<sup>lo</sup>, LECAM-1<sup>hi</sup>, CD45RA<sup>hi</sup>, CD45RB<sup>hi</sup>, CD45RO<sup>lo</sup> surface phenotype while effector Th cells are large and express the opposite phenotype CD44<sup>hi</sup>, LECAM-1<sup>lo</sup>, CD45RA<sup>lo</sup>, CD45RB<sup>lo</sup>, and CD45RO<sup>hi</sup> irrespective of the cytokines produced (Swain *et al*, 1990).

#### **1.4.3. The Basic Tenets of Th Cell Activation and Tolerization**

CD4 T cells can be signalled to undergo activation, tolerization (anergy) or deletion. The mechanisms of clonal deletion of T cells will be dealt with separately in Section 1.5. Under most circumstances, naive CD4<sup>+</sup> T cells become activated when they recognise their specific peptide bound to Class II MHC molecules on the surface of APCs (signal one) that express accessory molecules that can interact with co-receptors

on the T cells to deliver additional signals (signal two, costimulation) (Schwartz, 1990). Initially the responding cells secrete only IL-2, that drives proliferation, and are receptive to other factors that mediate their differentiation into Th1 or Th2 effector cells (see 1.4.5).

Costimulatory signals, in contrast to signal one, are neither antigen-specific nor MHC-restricted but are required for maximal T cell proliferation, cytokine production and induction of effector function. Antigen presentation in the absence of sufficient costimulation (Quill and Schwartz, 1987; Gimmi *et al*, 1993) or chronic stimulation via the TCR (DeSilva *et al*, 1991) results in anergy or tolerance - a long-lasting, but reversible, state characterised by antigen-specific proliferative unresponsiveness and a block in IL-2 production. These findings led to the classification of APCs into "professional" versus "non-professional" APCs. Dendritic cells, the most potent and costimulatory APC, are the archetypal professional APC (Steinman *et al*, 1991), whereas antigen presentation by resting B cells, that are considered non-professional APC because they lack costimulatory activity, induces T cell anergy (Eynon and Parker, 1992). Similarly, chemical fixation of antigen-pulsed APCs abolishes their costimulatory activity and renders antigen-specific T cells anergic (Harding *et al*, 1992).

A number of costimulatory pathways exist that augment the TCR-mediated signals (Kuhlman *et al*, 1991; Janeway and Bottomly, 1994; Boussiotis *et al*, 1994), the most well characterised being the interaction of CD28 with ligands of the B7 family (reviewed in Lenschow *et al*, 1996). Blocking this signalling pathway using CD28 antagonists prevents T cell activation and in some cases can induce anergy (Tan *et al*, 1993). Conversely, the addition of anti-CD28 MAb to T cell cultures with antigen-pulsed, chemically fixed APC, not only restores IL-2 driven proliferation but also prevents anergy induction (Harding *et al*, 1992). Without even considering other costimulatory pathways, the CD28/B7 interaction itself is increasingly complex. While



CD28 is expressed constitutively, levels increase on T cell activation and are then down-regulated to the constitutive level upon ligation. By contrast, CTLA-4, another T cell surface molecule that shares structural homology with CD28, binds both B7-1 and B7-2 with a 20-100 fold higher affinity than does CD28 (Linsley *et al*, 1994), is expressed only upon T cell activation, peaks at around 48 hours and then disappears from the surface. Therefore, the period when CD28 is down-regulated, and less responsive to signalling, coincides with maximal CTLA-4 expression (Lenschow *et al*, 1996). Although the function of CTLA-4 remains controversial, the majority of studies indicate that CTLA-4 acts as a negative regulator of T cell activation either directly (Walunas *et al*, 1994) or by blocking CD28-dependent costimulation (Walunas *et al*, 1996). Furthermore, members of the B7 family exhibit temporal differences in their expression that may be responsible for different biological effects (Hathcock *et al*, 1994). For instance, costimulators that are present on APC early after antigenic exposure (e.g. B7-2) may activate T cells, whereas costimulators that are expressed later (e.g. B7-1) may function to regulate the evolving T cell response (Freeman *et al*, 1993). However, the constitutive expression of B7-1 in transgenic mice inhibits T cell dependent responses when targetted to mature B cells (Sethna *et al*, 1994) but stimulates potent costimulation when expressed on pancreatic  $\beta$  cells (Guerder *et al*, 1994). Therefore, the outcome of the vast range of possible costimulatory interactions is likely to reflect the integration of signals that can be positive or negative depending primarily upon their temporal expression on different cell types.

The tight, reciprocal regulation of T cell activation and inactivation has important implications for the effective functioning of the immune system, that requires T cells to respond promptly against an infinitely diverse range of foreign antigens yet at the same time ignore potentially antigenic self-molecules.

Clonal deletion in the thymus is the major mechanism for establishing T cell self-tolerance. However, unresponsiveness towards self-antigens not expressed in, or that

cannot gain access, to the thymus is maintained, at least in part, by the induction of T cell anergy (Johnson and Jenkins, 1994). A number of examples of peripheral T cell unresponsiveness maintained by T cell anergy have been demonstrated *in vivo*. For example, in transgenic mice expressing Class II I-E MHC molecules exclusively on pancreatic  $\beta$  cells,  $V\beta 5^+$  and  $V\beta 14a^+$  T cells known to react with I-E associated superantigen (SAg) are unresponsive to *in vitro* stimulation with anti-TCR antibody. However dual expression of I-E and B7 transgenes in pancreatic  $\beta$  cells results in insulinitis and diabetes. Therefore, antigen-presentation by a B7-negative tissue results in tolerance whereas antigen presentation by a B7-positive tissue results in activation, autoimmunity in this case (Guerder, 1994).

It has also been known for many years that certain routes of administration of soluble protein antigen results in immunity versus unresponsiveness, as assessed by *in vitro* re-simulation assays. To investigate the cellular mechanisms responsible for this phenomenon, a small number of OVA peptide-specific TCR-transgenic T cells were adoptively transferred into a syngeneic recipient and the fate of this small population, after antigenic challenge, was followed *in vivo* using a clonotypic TCR MAb (Kearney *et al*, 1994). Using this novel technique, it was shown that when OVA is injected subcutaneously in complete Freund's adjuvant (CFA), antigen-specific T cells proliferate in the lymph nodes and move to the primary follicles. They then disappeared slowly from the draining nodes and the remaining cells were hyperactive to restimulation (i.e. memory Th cells). In contrast, when OVA is injected alone, either i.v. or i.p., antigen-specific T cells proliferate for a short time but never entered follicles. Most of the cells rapidly disappeared from the draining nodes, and the small number of remaining cells were hyporesponsive to antigenic stimulation. This contrasting outcome has been attributed to the presentation of antigen by professional (s.c.) versus non-professional APC (i.v. and i.p.). In addition, the bacterial components in the CFA are known to further enhance the costimulatory function of APCs, in part by inducing the expression of B7 molecules (Freeman *et al*, 1993).

Conversely, the strict requirements for activation are thought to be responsible for the observation that only professional APC can activate naive CD4 cells (Croft *et al*, 1992). As mentioned above, autoreactive T cells that persist will therefore exist in an anergised state, since the majority of self-antigens are expressed on non-APCs. In addition, undeleted, autoreactive T cells should not be activated by the presentation of self-antigens by dendritic cells since other cell types cannot deliver signal two. It therefore follows that naive CD4 T cells should respond only when dangerous antigens are presented by professional APCs (Matzinger, 1994). Unlike the intravenous presentation of soluble, monomeric antigen, foreign antigens are more likely to appear in a multi-valent and aggregated form that stimulates the costimulatory activity of APCs. Furthermore, foreign antigens are also more likely to enter the body at sites (e.g. lungs and skin) where potent APCs like dendritic cells and activated macrophages are situated to pick-up, process and transport them to the local lymph node for presentation to T and B cells. Therefore, primary immune responses to foreign antigens can be rapidly initiated. It has recently become clear that effector cells require less costimulation than naive cells (Dubey *et al*, 1996). This may explain how effector cells that have to relocate to the site of infection, where foreign antigens may then be presented by non-professional APCs, are able to continue to expand and function as long as the pathogen persists.

However, there is an abundance of evidence, mentioned later, that foreign organisms, in particular parasites, appear to have evolved mechanisms to suppress and/or tolerize effector immune mechanisms that would result in their destruction. In this respect, it is interesting to note that Th1 and Th2 cells may have different costimulatory requirements at the level of their differentiation from naive cells (See Section 1.4.5) and their effector function (See Section 1.4.6) and therefore, costimulatory signals may function as an additional regulator of Th responses.

The development of variants of antigenic peptides (altered peptide ligands, APL, generated by single aa substitutions of the critical TCR contact residues) has revealed a further level of complexity in T cell activation and the resulting effector functions based on the ability of the TCR to partially signal (Evavold *et al*, 1993). APLs bind MHC molecules with the same affinity as the "wild-type" peptide, but are perceived by the TCR in a subtly different way. Recognition of APLs can result in cytokine production without proliferation (Evavold and Allen, 1991), a change in cytokine profile (Windhagen *et al*, 1995) or tolerance (Vidal *et al*, 1996). The general conclusion from these studies is that naturally occurring APLs are able to modulate TCR-mediated effector functions by partial stimulation. Therefore, for example, memory Th cells may be maintained in the periphery in the absence of foreign antigen by constant engagement of weak ligands that can only partially stimulate.

#### **1.4.4. Parasitic Infections as Models for the Study of CD4<sup>+</sup> T Cell Development and Regulation**

Parasitic infections tend to result in polarized Th1 versus Th2 cell responses and as such have proved invaluable model systems for analysing several important questions relating to Th cell subset development and regulation. What early events in infection initiate the subsequent polarised response? How does the established response remain stably polarised? Three of the most well-characterised model parasites are *L. major*, *Schistosoma mansoni* and *Trichuris muris*, which can fully develop in particular inbred mouse strains.

In certain inbred strains of mice, it has been possible to correlate susceptibility and resistance with the development of a particular Th cell phenotype. Protection against *T. muris* and other intestinal nematodes requires Th2 activation while a Th1 response is associated with chronic infection (Else *et al*, 1992; Grencis *et al*, 1992; Urban *et al*,

1992); the opposite appears to be the case for intracellular pathogens, including *L. major* (Heinzel *et al*, 1989; Gazzinelli *et al*, 1993; Hsieh *et al*, 1993).

In murine trichuris, BALB/k mice, that expel the parasite rapidly (before the development of the third stage larvae), produced elevated levels of IL-4, IL-5, and IL-9 and very low levels of IFN- $\gamma$ . In addition, resistant mice exhibited a significant intestinal mastocytosis and increased levels of parasite-specific serum IgG1 and total IgE. In contrast, B10.BR mice, that are unable to expel the parasite before the infection reaches patency, produced large amounts of IFN- $\gamma$  in the relative absence of IL-4, IL-5 and IL-9 and increased levels of parasite-specific serum IgG2a (Else *et al*, 1990; Else and Gencis, 1991; Else *et al*, 1992). It is considered that the Th2-mediated IgE-mast cell-eosinophil response is the major effector mechanism that functions against nematode worms (Finkelman *et al*, 1991). However, additional mechanisms are likely to exist (Locksley *et al*, 1994) to explain, for example, why antibodies to IL-3, IL-4, IL-5 and IL-9, that abrogate mast cell hyperplasia, eosinophilia and IgE production, have no effect on the course of infection of normal mice with the intestinal nematode *Nippostrongylus brasiliensis* (Madden *et al*, 1991; Coffman *et al*, 1989) and also why IL-4, administered to SCID mice, is able to confer resistance to infection with *N. brasiliensis* (Urban *et al*, 1995).

In contrast, infection of susceptible BALB/c mice, that develop an uncontrolled and ultimately fatal disease, with *L. major* induced sustained IL-4 transcription in the draining popliteal lymph nodes. However, resistant mouse strains, such as C3H, CBA and C57BL/6, that heal their lesions, express transcripts for IFN- $\gamma$  but not IL-4 (Heinzel *et al*, 1989). This is consistent with the increased production of IL-4 protein (Scott, 1991) and frequency of IL-4 secreting precursor T cells in lymph node cells of BALB/c mice one week after infection (Morris *et al*, 1992). Induction of a Th1 response controls the replication of the leishmania parasite through the production of macrophage activating cytokines like IFN- $\gamma$ , while the induction of a Th2 response is unable to

mediate macrophage activation. Nitric oxide-dependent killing of *L. major* by macrophages is stimulated by IFN- $\gamma$  and inhibited by IL-4 (Liew *et al*, 1990; Sher *et al*, 1992). Furthermore, parasite-specific Th1 but not Th2 cell lines can transfer resistance (Holaday *et al*, 1991; Scott *et al*, 1988).

In murine schistosomiasis, the correlation between Th subset induction with host protection is not quite as simple. Mice are not naturally resistant and, unlike intestinal nematode infections, *S. mansoni* adults are tissue-dwelling and their eggs can become trapped in the liver. In addition, different stages induce different Th responses; chronic infection is associated with an anti-egg Th2 response while immunisation with irradiated cercariae results in a protective Th1 response triggered primarily by larval antigens (Pearce *et al*, 1991).

#### **1.4.5. Factors Influencing the Pathway of Th Differentiation**

Although theoretically Th1 and Th2 cells may arise from distinct precursors, a number of studies have confirmed the ability of naive, uncommitted IL-2 producing CD4<sup>+</sup> T cells to differentiate into either Th1 or Th2 effector cells (Street *et al*, 1990; Rocken *et al*, 1992; Kamogawa *et al*, 1993). Various factors have been implicated in triggering the development of a particular Th cell response. In this respect,  $\alpha\beta$  T cell receptor (TCR) transgenic mice have been an important innovation, as a source of naive CD4<sup>+</sup> T cells with a defined antigen-specificity that can be activated *in vitro* under well-defined conditions of APC, cytokines and exposure to pathogens.

##### **1.4.5.1. Cytokines**

Many studies, both *in vivo* and *in vitro*, have indicated that cytokines present at the time of T cell priming mediate Th cell differentiation. The vital cytokines are considered to be IL-4 and IL-12, that direct Th2 versus Th1 development respectively, although IFN- $\gamma$  and IL-10 have also been shown to exert some, if not a critical, influence.

## IL - 4

The first evidence that IL-4 was essential for Th2 priming came from studies demonstrating that the presence of IL-4 during initial priming with mitogen or re-stimulation with specific antigen gives rise to IL-4 producing cells (Swain *et al*, 1990a; Betz and Fox, 1990) and suppresses IL-2 and IFN- $\gamma$  production (Tanaka *et al*, 1993). These results were confirmed using naive CD4<sup>+</sup> T cells from TCR transgenic mice specific for OVA (Hsieh *et al*, 1992) or for pigeon cytochrome c (Seder *et al*, 1992). Both studies found that the presence of IL-4 during primary stimulation resulted in a cell population that produced IL-4 upon subsequent re-stimulation, and that the appearance of IFN- $\gamma$ -producing cells was suppressed.

These results are supported by the observation that *in vivo* treatment with anti-IL-4 at the time of infection with *L. major* (Chatelain *et al*, 1992), *T. muris* (Else *et al*, 1994), *Candida albicans* (Romani *et al*, 1992) or immunisation with haemocyanin (Gross *et al*, 1993) markedly diminished the appearance of Th2 cells. Furthermore, infection of IL-4 KO mice with the helminth parasites *N. brasiliensis* (Kopf *et al*, 1993), *B. malayi* (Lawrence *et al*, 1995) or *S. mansoni* (Pearce *et al*, 1995) resulted in impaired Th2 responses. Conversely, the constitutive expression of IL-4 in transgenic mice of a *L. major* resistant background renders them susceptible to infection following activation of a Th2 response (Leal *et al*, 1993). Comparison of early cytokine mRNA production after *L. major* infection demonstrated that, in both resistant and susceptible strains, CD4<sup>+</sup> cells from the draining LN contained transcripts for IL-4 and IFN- $\gamma$  at day 4 p.i. However, IL-4 expression by CD4<sup>+</sup> T cells was then rapidly down-regulated in resistant strains but remained elevated in susceptible strains (Reiner *et al*, 1994). Furthermore, in addition to anti-IL-4, treatment with anti-CD4 (Titus *et al*, 1985), anti-IL-2 (Heinzel *et al*, 1993), IL-12 (Heinzel *et al*, 1993a) or blockade of CD28-B7 interaction using CTLA-4 Ig (Corry *et al*, 1994) were all capable of reversing the response of BALB/c mice to a curative Th1 phenotype. The unifying effect of all these

immunological interventions was to attenuate the expression of IL-4 consistent with the important role for IL-4 in mediating Th2 development *in vivo*.

However, studies involving infection of IL-4 KO mice with *L. major* have done little to clarify the role of IL-4 in mediating susceptibility in that model. Using the same genetically pure BALB/c IL-4 KO mice to study the outcome of infection with *L. major*, two separate studies have arrived at opposite conclusions. Noben-Trauth *et al* (1996) demonstrated that IL-4 KO mice remain susceptible to *L. major* infection, and exhibited no change in IL-10, IFN- $\gamma$  or IL-12 cytokine production compared to wild-type controls. However, although susceptible to infection there was some decline in parasitemia and lesion size in IL-4 KO mice in comparison to wild-type controls, that were still progressing at later time points. In contrast, in the study by Kopf *et al* (1996), IL-4 KO mice were fully resistant to progressive infection and showed no signs of necrosis of the lesion. However, while Th2 cytokine production was impaired in IL-4 KO mice (8-16x less IL-10 and IL-13 in IL-4 KO mice v wt controls) there was no preferential expansion of Th1 cells (IFN- $\gamma$  and IL-12 transcripts were comparable in IL-4 KO v wt controls). Since the induction of inflammatory cytokines and iNOS was also unaltered in the absence of IL-4, the mechanism of resistance in IL-4 KO mice was not apparent. However, heterozygotes exhibited smaller lesions, less ulceration and necrosis than wt controls indicative of gene-dosage effect and therefore the magnitude of the IL-4 response appears to determine the severity of disease following *L. major* infection. The discrepancy in the results between these two studies may be attributable to the different strains of *L. major* used.

## IL - 12

IL-12 appears to be the dominant factor in promoting Th1 cell generation in mice and humans by acting on two levels; by direct costimulation of T cells and by enhancing production of IFN- $\gamma$  from NK and T cells which serves to further stimulate Th1 differentiation by an autocrine mechanism (Scott, 1993; Schmitt *et al*, 1994). CD4<sup>+</sup> T



cells from TCR transgenic mice differentiated into IFN- $\gamma$  producing cells when IL-12 was added to priming cultures stimulated with specific antigen and APC (Hsieh *et al*, 1993). Similarly, in cultures of highly purified CD4 T cells from normal mice with immobilized CD3 and without APC, IL-12 strikingly enhanced priming for IFN- $\gamma$  producing cells and diminished IL-4 inhibition of such priming (Seder *et al*, 1993; Schmitt *et al*, 1994a). In addition, IL-4 can inhibit the ability of IL-12 to promote Th1 cell development (Hsieh *et al*, 1993). Therefore, the balance of IL-12 and IL-4 present during priming appears to be critical in driving the lineage towards the Th1 or Th2 cell pathway respectively. However, when IL-12 and IL-4 are present together *in vitro*, the effect of IL-4 is dominant since the emerging T cells are IL-4 producing (Hsieh *et al*, 1993; Schmitt *et al*, 1994a), although production of IFN- $\gamma$  is gradually restored when the addition of IL-4 is delayed (Schmitt *et al*, 1994a).

A recent study by Manetti *et al* (1993) demonstrated that IL-12 may have a similar role in the differentiation of Th1 cells in humans. When rIL-12 is added to PBMC cultures from atopic patients, Th cell lines with a Th1 like phenotype develop whereas in the absence of IL-12, Th2-like lines are generated. Conversely, the addition of anti-IL-12 to cultures of PBMCs stimulated with bacterial antigen induced the generation of Th2-like lines and clones whereas in the absence of antibody Th1-like lines would develop. Furthermore, IL-12 has been shown to induce the production of IFN- $\gamma$  by antigen-inexperienced neonatal human CD4 T cells stimulated with immobilized anti-CD3 (Wu *et al*, 1993).

These data are consistent with *in vivo* studies which showed that the ability of several intracellular pathogens and their products to induce a Th1 response correlates with their ability to produce IL-12 (D'Andrea *et al*, 1992; Scott, 1993; Hsieh *et al*, 1993; Gazzinelli 1993). Furthermore, treatment of BALB/c mice with rIL-12 at the time of or within the first week of infection with *L. major* induced a protective Th1 response, while treatment of resistant C57BL/6 mice with anti-IL-12 reduced IFN- $\gamma$  production

and exacerbated disease (Sypek *et al*, 1993). In addition, immunisation with soluble leishmania antigen and rIL-12 induces a protective Th1 response in BALB/c mice indicating a role for IL-12 as a "Th1 adjuvant" (Afonso *et al*, 1994). Similar experiments have been performed using the murine model of schistosomiasis. rIL-12 administered at the time of s.c. injection with eggs inhibits Th2 responses by the induction of IFN- $\gamma$  (Oswald *et al*, 1994). In addition, anti-IL-12 increases, and IL-12 protects against, Th2-associated granuloma formation (Wynn *et al*, 1994). IL-12 has also been used in conjunction with *S. mansoni* eggs (Wynn *et al*, 1994), irradiated cercariae (Wynn *et al*, 1995) or soluble larval antigen (Mountford *et al*, 1996) to stimulate a protective anti-parasite Th1 response.

Recently, it has been suggested that the maintenance of responsiveness of T cells to IL-12 may be an intrinsic property that is the critical factor for the stable commitment to a Th cell phenotype (Güler *et al*, 1996). Previously, the same group had argued that strain differences in the generation of distinct Th responses to the same stimulus, may reflect a genetically determined default pathway that resides within the T cell, not the APC compartment (Hsieh *et al*, 1995). A genetically determined Th pathway is consistent with the *in vivo* Th responses of BALB/c (Th2) and B10 (Th1) mice to infection with *L. major* and *T. muris*. Further analysis revealed that the IL-12 signalling pathway can be blocked during Th2 development (Szabo *et al*, 1995) and that T cells derived from BALB/c, but not B10.D2 TCR Tg mice, rapidly lose their ability to respond to IL-12 (Güler *et al*, 1996). Since the Th2 cells generated in the study by Szabo *et al* (1995) retained the capacity to respond to IL-2, IL-4, IFN- $\gamma$  and IFN- $\alpha$ , which are known to share at least some common signalling elements with IL-12, the defect in Th2 cells for IL-12 responses was traced back to some component of the IL-12 receptor itself. The expression of IL-12 receptors and their binding affinity for IL-12 was identical on Th1 and Th2 cells, therefore component of the IL-12 receptor responsible for the defect in Th2 cells, in this system, is as yet unidentified. However, ruling out the importance of IL-4 in Th priming is clearly an over-simplification. IL-12 production is delayed for

several days after *L. major* infection, in both resistant and susceptible mice, during which time an IL-4-induced Th2 response is established in BALB/c mice and IL-4 expression is down-regulated in resistant mice (Reiner *et al*, 1994). In addition, BALB/c mice can develop stable antigen-specific Th1 cells *in vitro* (Szabo *et al*, 1995) and *in vivo* (Sypek *et al*, 1993), even if IL-12 is present initially.

## IFN- $\gamma$

While evidence exists that IFN- $\gamma$  enhances Th1 development, the essential requirement for IFN- $\gamma$  in the priming of naive T cells into Th1 cells is uncertain. Treatment of *T. muris* susceptible mice with anti-IFN- $\gamma$  resulted in depressed IgG2a levels, elevated IgG1 production and the expulsion of adult worms indicative of Th2 activation (Else *et al*, 1994). In addition, a single administration of anti-IFN- $\gamma$  to a *L. major* resistant strain promotes susceptibility to infection by reducing Th1 development (Scott, 1991) and both IFN- $\gamma$  and IFN- $\gamma$  receptor KO mice fail to clear *L. major* infection (Wang *et al*, 1994; Swihart *et al*, 1995). Furthermore, the presence of IFN- $\gamma$  during the generation of T cell clones results in the almost exclusive production of IFN- $\gamma$  secreting Th1 clones (Gajewski *et al*, 1989). However, it is possible that Th1 clones emerge because the exogenous IFN- $\gamma$  inhibits the proliferation of IL-4 producing cells rather than stimulating naive cells to differentiate into Th1 cells. A single or the sustained administration of rIFN- $\gamma$  to BALB/c mice, while transiently reducing IL-4 expression, was unable to reverse the ultimate progressive course of disease (Scott, 1991). In addition, while IFN- $\gamma$  KO mice and IFN- $\gamma$  receptor KO mice are both susceptible to *L. major* infection (Wang *et al*, 1994; Swihart *et al*, 1995), only IFN- $\gamma$  KO mice produce high levels of IL-4 and little TNF- $\beta$ ; IFN- $\gamma$  receptor KO mice produce high levels of IFN- $\gamma$  and little IL-4 following infection. The fact that IFN- $\gamma$  receptor KO mice display susceptible phenotype in the absence of a Th2 responses is thought to be due to the genetic background of the mutant mice. While IFN- $\gamma$  KO mice have been backcrossed seven generations onto the C57BL/6 background, IFN- $\gamma$  receptor KO mice were bred on a 129/Sv/Ev background. Despite both wild-type strains being intrinsically resistant,

wild-type 129/Sv/Ev treated with anti-IFN- $\gamma$ , failed to develop a Th2 response in contrast to treated C57BL/6 mice.

*In vitro* studies have been just as conflicting. Swain *et al* (1990a) reported that the presence of IFN- $\gamma$  (only at very high levels) in the priming culture of naive CD4<sup>+</sup> T cells with mitogen and APC enhanced the subsequent development of Th1 cells. In addition, in one TCR Tg mouse model (BALB/c background), the presence of anti-IFN- $\gamma$  antibody in the initial culture diminished the appearance of IFN- $\gamma$  producing cells in the secondary culture, although the addition of IFN- $\gamma$  did not enhance priming for IFN- $\gamma$  production (Macatonia *et al*, 1993). However, the results from another TCR Tg model (B10.A background) showed that the presence of anti-IFN- $\gamma$  did not reduce Th1 priming nor did the addition of IFN- $\gamma$  increase priming for IFN- $\gamma$  production (Seder *et al*, 1992). Therefore, Th1 priming of naive CD4<sup>+</sup> T cells from TCR Tg mice on a BALB/c background, but not B10.A, was diminished by treatment with anti-IFN- $\gamma$  (Seder *et al*, 1992; Macatonia *et al*, 1993), consistent with the intrinsic ability of naive T cells from these mouse strains to develop a Th2 and Th1 phenotype, respectively (Hsieh *et al*, 1995). The conclusion from these studies was that IFN- $\gamma$  alone is insufficient to induce Th1 differentiation. However, anti-IFN- $\gamma$  can reduce the effect of IL-12 on Th1 priming (Schmitt *et al*, 1994), indicating that IFN- $\gamma$  plays an important role in Th1 development, through its induction by IL-12. In addition, it has recently been discovered that IFN- $\alpha$  can replace the requirement for IFN- $\gamma$  which may in part explain the discrepancies mentioned above (Wenner *et al*, 1996).

## **IL - 10**

The evidence for the involvement of IL-10 in Th priming is also conflicting. Three separate studies using T cells from TCR Tg mice and different sources of APC have presented confusing results. One study showed that the addition of IL-10 to priming cultures, in which spleen cells were used as APC, diminished priming for IFN- $\gamma$  production and that neutralisation of endogenous IL-10 enhanced the ability of T cells

to produce IFN- $\gamma$  upon restimulation (Hsieh *et al*, 1992). However, the effect of IL-10 is not dependent on spleen cells as APCs, since IL-10 significantly reduced the amount of IFN- $\gamma$  produced upon restimulation when highly purified dendritic cells were used as APC (Macatonia *et al*, 1993). This is in contrast to the study by Seder *et al* (1992) in which the addition of IL-10 or anti-IL-10 to priming cultures, in which the APCs were purified dendritic cells or activated B cells, had no effect on IFN- $\gamma$  priming of naive T cells.

#### **1.4.5.2. Antigen**

##### **Type**

Injection of mice with certain antigens has been shown to preferentially elicit Th1 or Th2 like responses (Yang *et al*, 1993; Baum *et al*, 1990; DeWit *et al*, 1992). For example, high doses of soluble antigen, administered without adjuvant, selectively suppresses Th1 cells, leaving Th2 responses unaffected or enhanced (Burstein *et al*, 1992; Burstein and Abbas, 1993; Karpus *et al*, 1994; DeWit *et al*, 1992). The resulting Th1 unresponsiveness is thought to be due to a combination of clonal T cell anergy, as a result of antigen presentation by resting B cells and macrophages which lack necessary costimulatory signals (Eynon and Parker, 1992), and Th2 mediated immune-regulation (Burstein and Abbas, 1993). In a recent study, an OVA peptide-specific DO11.10 TCR Tg mouse model was used for a more direct analysis of the role of soluble antigen administration in Th cell differentiation from a naive clonal CD4<sup>+</sup> population (Degermann *et al*, 1996). Using an anti-clonotypic MAb to follow Tg T cells that were adoptively transferred to normal BALB/c mice primed with OVA in CFA, demonstrated that the naive Tg T cells expand and differentiate into Th1 cells producing IL-2 and IFN- $\gamma$ . In contrast, if recipient mice are primed with soluble OVA, the Tg T cells do not proliferate or secrete IL-2 and IFN- $\gamma$ , when re-stimulated *in vitro*, but produce IL-4 and IL-5. Therefore, administration of soluble antigen itself by appears to divert the immune response of a naive, clonal CD4<sup>+</sup> T cell population in the Th2 direction.

In the *Leishmania* model, Th1 and Th2 cell lines that confer resistance v susceptibility to infection, appear to recognise separate antigens (Scott *et al*, 1988), suggesting that Th1-specific epitopes could be identified that may be used as potential vaccine candidates. Initial studies, using peptide epitopes of a conserved membrane protease gp63, demonstrated protection of BALB/c mice when administered with adjuvant (Jardim *et al*, 1990). However, an additional study was unable to demonstrate that immunisation with this peptide was able to protect BALB/c mice against a higher dose of a different strain of *L. major* (Wang *et al*, 1993). Similar studies identified, an octamer of a 10-residue peptide (corresponding to the tandemly repeating regions of *Leishmania* proteins) that preferentially induces Th2 cells when administered subcutaneously to susceptible BALB/c mice and caused exacerbation of subsequent infection (Liew *et al*, 1990a). Since such studies are complicated by differences in antigen processing and presentation in different mouse strains, it was decided to investigate whether there were distinct parasite antigens capable of eliciting protective v nonprotective responses by analyzing TCR usage at early time points in normal and anti-IL-4-treated BALB/c mice and in C57BL/6 mice (Reiner *et al*, 1993). Expansion of a restricted population of CD4<sup>+</sup> T cells expressing V $\alpha$ 8-J $\alpha$ TA72, V $\beta$ 4 was identified in all groups suggesting that an immunodominant parasite epitope is driving opposing Th outcomes. Therefore, in murine leishmaniasis, antigens that drive the CD4<sup>+</sup> response are not the major determinants of Th1 or Th2 commitment. However, this may not be the case for parasitic helminth infections that preferentially induce Th2 responses. For example, the oligosaccharide LNFPIII found on schistosome egg glycoproteins can directly induce IL-10 production from B cells (Velupollai and Harn, 1994). Two recombinant antigens of *O. volvulus*, Ov27 (a cysteine protease inhibitor) and OvD5B (an aspartyl protease inhibitor) were shown to induce PBMCs, from patients infected with *Loa Loa* or *O. volvulus*, to produce IgG4 and IgE antibodies and IL-4, IL-5 and IL-10 (but not IFN- $\gamma$ ) (Garraud *et al*, 1995). In addition, a recombinant protein from *Brugia*, termed gp15/400, that is homologous to an allergen produced by *Ascaris suum*

and *A. lumbricoides*, is recognised by IgE antibodies in individuals infected with *B. malayi* (Paxton *et al*, 1993). However, the search for other Th2 polarizing antigens derived from helminth parasites has not proved very successful. APLs that are known to modulate peripheral T cell responses due to changes in the TCR-peptide/MHC interaction (Vidal *et al*, 1996), may also influence the differentiation of naive CD4<sup>+</sup> T cells (Pfeiffer *et al*, 1995) (see below).

### **Dose-Peptide/MHC Interaction**

It has recently been demonstrated that differentiated CD4<sup>+</sup> T cells will produce different cytokines depending on the dose of antigen used (Secrist *et al*, 1995; Carballido *et al*, 1992). For many years there has been speculation as to whether antigen dose or ligand density can directly induce the development of Th1 or Th2 cells from naive precursors.

Pfeiffer *et al* (1991) demonstrated that human type IV collagen was capable of eliciting a Th1-type response in H-2<sup>s</sup> mice and a Th2-type response in H-2<sup>b</sup> mice that both respond to the same 12aa peptide. While this may reflect different T cell repertoire in the two mouse strains, the authors preferred the explanation that the effective concentration of type IV collagen peptide/MHC complexes was different depending on the MHC type, possibly because of differences in the binding efficiencies of different MHC molecules. In support of this hypothesis, the same group have recently demonstrated, using APLs derived from the 12 aa human type IV collagen peptide, that peptide/MHC complexes that interact strongly with the TCR favour the generation of Th1-like cells while those that bind weakly favour priming of Th2-like cells (Pfeiffer *et al*, 1995).

With the development of TCR Tg mice, the effect of peptide dose on the activation of naive CD4<sup>+</sup> T cells with the same T cell receptor could be investigated directly. In one model (OVA peptide specific I-A<sup>d</sup>-restricted DO11.10 TCR  $\alpha\beta$  mice on a BALB/c

background) (Hosken *et al*, 1995) initial priming in the presence of low-to-medium doses of antigen (0.3-0.6  $\mu$ M) results in the appearance of Th1 cells producing IFN- $\gamma$  and undetectable levels of IL-4. In contrast, very high ( $>10\mu$ M) and very low ( $<0.05\mu$ M) doses of antigen stimulates the priming of naive T cells into Th2-like cells producing IL-4. This is coincident with massive cell death in the cultures suggesting that a contaminating population of committed IL-4 producing cells with enhanced viability may exist. However, the same results were obtained when highly purified CD4<sup>+</sup> LECAM-1<sup>hi</sup> T cells were used. Neutralization of endogenous IL-4 in priming cultures, completely inhibited the development of IL-4 producing Th2 cells at very low and very high antigen doses. These data suggest that the antigen dose may influence the development of Th phenotype by affecting the level of IL-4 in primary cultures. In another model (pigeon cytochrome-c peptide specific I-E<sup>k</sup>-restricted 5C.C7 TCR  $\alpha\beta$  mice on a B10.A background) initial priming in the presence of low doses of antigen (0.5-5ng/ml) induced Th2 differentiation, while high doses ( $>50$ ng/ml) enhanced priming for IFN- $\gamma$  producing Th1 cells by subsequent re-stimulation (Constant *et al*, 1995). Again the same results were obtained when highly purified CD4<sup>+</sup> LECAM-1<sup>hi</sup> T cells were used. However, neutralization of endogenous IL-4 in low antigen dose priming cultures, had no effect on the production of IL-4 upon secondary re-stimulation. Therefore, in this model it is not clear how the dose of antigen influences Th cell selection.

Since antigen-specific T cells in normal mice generally exist at a much lower level than in TCR Tg models, it remains to be determined how antigen dose may influence Th differentiation in physiological responses *in vivo* where, in combination with several other Th polarizing factors, various peptide/MHC complexes are presented to be recognised at different affinities by a wide range of T cells. In this respect some interesting *in vivo* observations have been made. For example, administration of a single low dose of *L. major* renders susceptible BALB/c mice resistant to challenge with a larger inoculum (Bretsher *et al*, 1992) and a single low dose infection with 40



*T. muris* eggs results in the development of sexually mature adults and coincident Th1 response in normally resistant BALB/k Th2 responder mice (Bancroft *et al*, 1994). Therefore, in two models, it appears as if exposure to lower level infection results in the generation of Th1 responses. However, trickle infection with low numbers of *T. muris* eggs results in a protective Th2 response in BALB/k mice (Bancroft and Grencis, pers. comm.). Similarly, the continuous administration of low dose soluble protein antigen (delivered by a mini-osmotic pump over a day 10 period) preferentially stimulates Th2 responses (Guéry *et al*, 1996). However, there is a danger in attempting to correlate the effect obtained with different doses of simple proteins with that induced by complex organisms, where other factors may dominate. For example, the susceptibility of BALB/k mice to low dose *T. muris* infection can be attributed to the fact that the larval stages of *T. muris* are immunogenic while the adults are immunomodulatory and therefore antigen dose relates to a threshold level of antigen that is required to elicit a strong enough Th2 response to prevent development of adult worms.

#### **1.4.5.3. APC**

The demonstration that macrophage derived IL-12 enhances priming of naive CD4<sup>+</sup> T cells for IFN- $\gamma$  production (Hsieh *et al*, 1993; Macatonia *et al*, 1995) is a clear example of the influence of APC on Th priming that is attributable to cytokine production. A similar function for APC-derived IL-10 in Th2 differentiation is less clear, as mentioned previously. In addition, IL-4 can direct the development of Th2 cells from naive CD4<sup>+</sup> T cells in TCR Tg mice, independent of the APC used, including dendritic cells, activated B cells, and I-E<sup>k</sup>-transfected fibroblasts (Seder *et al*, 1992; Hosken *et al*, 1995). However, more recently it has been shown that exposure of macrophages to IL-4 and IL-10 *in vivo* before the initial encounter with antigen results in activation of Th2 cells, and no Th1 priming, from naive precursors (Cua *et al*, 1996). Furthermore, since the monocytes secrete prostaglandin-E2 (PGE<sub>2</sub>), in addition to IL-12, during antigen presentation, the balance between these two soluble factors may also play a role in determining Th differentiation. PGE<sub>2</sub> inhibits the production of IL-12 from monocytes

(van der Pouw Kraan *et al*, 1995) and IFN- $\gamma$  from Th1 cells but not Th2 cytokines IL-4 and IL-5 from Th2 cell lines (Betz and Fox, 1991).

A number of studies have indicated that CD28-mediated signalling via APC costimulatory receptors directly regulates Th1/2 differentiation. It has been demonstrated that in the absence of CD28-signalling (using CTLA-Ig), antigen-stimulated, naive TCR Tg T cells were biased towards a Th1 phenotype (Seder *et al*, 1994), whereas anti-CD28 ligation resulted in IL-4 and IL-5 production by naive human T cells, even in the absence of IL-4 (King *et al*, 1995). Furthermore, CD28 KO mice have a reduced Th2-dependent antibody responses while Th1 dependent DTH responses remain intact (Shahinian *et al*, 1993). In addition, treatment of mice with CTLA4-Ig at the time of infection with *H. polygyrus* (Lu *et al*, 1994) or *L. major* (Corry *et al*, 1994) effectively abrogated Th2 responses while sparing Th1 function. It has also been suggested that B7-1 and B7-2 directly control Th1 v Th2 development. The presence of anti-B7-2 MAbs in cultures of naive TCR Tg T cells stimulated with Ag resulted in IFN- $\gamma$  production, while the presence of anti-B7-1 MAbs increased IL-4 secretion (Kuchroo *et al*, 1995). However, it has also been shown that B7-1 and B7-2 can provide costimulatory signals for both Th1 and Th2 development (Lanier *et al*, 1995).

Furthermore, recent studies have disputed an absolute role for CD28-signalling in driving Th2 differentiation. Firstly, treatment with CTLA-4 Ig inhibits Th1 responses, but not Th2 responses, in allograft transplantation (Sayegh *et al*, 1995). Furthermore, in response to *L. major* infection, C57BL/6 CD28 KO mice exhibit normal Th1 responses and are resistant, while BALB/c CD28 KO mice unexpectedly exhibited normal susceptibility and expressed high levels of IL-4 (Brown *et al*, 1996). In addition, when naive CD28<sup>+/+</sup> TCR Tg CD4 T cells from BALB and C57BL backgrounds were activated *in vitro* in the presence of rIL-4 and CTLA-Ig, BALB T cells retained the ability to produce IL-4 and at a level much greater level than that of C57BL T cells

(Brown *et al*, 1996). This is consistent with the demonstration that the genetic background determines the default Th phenotype pathway in the absence of other polarizing signals, so that T cells from a BALB background acquire a stronger Th2 phenotype than T cells from a B10 background, that instead develop in the Th1 direction (Hsieh *et al*, 1995). Therefore, the B10 background of the TCR Tg mice used in the study by Seder *et al* (1994), referred to above, may explain why the presence of CTLA-4 Ig, under neutral priming conditions, had no effect on Th1 phenotype development.

The conclusion from these various studies is that while engagement of the CD28 pathway is important in T cell activation and can even select for Th2 cell development under certain circumstances, CD28-mediated signals may have a limited role in either Th1 or Th2 cell differentiation when other Th polarizing factors are dominant. Therefore, factors determined by genetic background and features of the foreign immunogen may obviate the need for costimulation. In addition, the discovery of novel Th1 costimulatory molecules (Cocks *et al*, 1995), suggests a level of redundancy in both Th differentiation pathways.

### **Summary: Factors That Influence Th Phenotype Commitment**

Together these findings support a complex model of Th maturation in which there are numerous distinct signals sufficient to drive Th2 differentiation, yet no signal is an absolute requirement in the presence of the other determinants. Cytokine milieu, genetic background, antigen dose, route of administration, APC function or level of costimulation all appear to influence Th differentiation. In addition, the mode of action of the different factors can overlap in that, for example, the effect of route may be dependent on the costimulatory function of the initiating APC population which in turn can produce or receive distinct cytokine signals, all of which could influence Th cell differentiation. Therefore, the individual contribution of different factors is likely to differ depending upon the presence of other factors during the priming event. Thus, for

example, under conditions of low antigen density, that appears to select for Th2 cell differentiation (Hosken *et al*, 1995; Constant *et al*, 1995), CD28 ligation may become important (Bluestone *et al*, 1995) whereas the presence of high levels of IL-4 can drive Th2 cell development regardless of the APC population present (Seder *et al*, 1992; Hosken *et al*, 1995).

#### **1.4.6. Factors Influencing the Function of Th Effector Cells**

Evidence from *in vitro* studies indicate that IL-4, IL-10, IFN- $\gamma$ , IL-12 and TGF- $\beta$  are the predominant cytokines that regulate Th1 and Th2 effector cells. IL-10 was first identified as a product of Th2 clones that inhibited production of IFN- $\gamma$  by Th1 clones (Fiorentino *et al*, 1989). IL-4 inhibits IFN- $\gamma$  production by human PBMCs (Peleman *et al*, 1989) and synergises with IL-10 to inhibit DTH responses including IFN- $\gamma$  production *in vivo* (Powrie *et al*, 1993). Conversely, IFN- $\gamma$  inhibits the growth of Th2 clones (Gajewski and Fitch, 1988).

APCs have been shown to exhibit a differential ability to stimulate the proliferation of Th clones. It was shown that while Th1 and Th2 clones proliferated in response to whole spleen cells as a source of APC, Th1 clones responded optimally when adherent spleen cells were used and Th2 clones proliferate maximally when splenic B cells were the source of APC (Gajewski *et al*, 1991). Similarly, CD4<sup>+</sup> T cells from allergic patients produce higher levels of IL-4 upon *in vitro* stimulation with allergen, when B cells rather than monocytes are used as APC (Secrist *et al*, 1995). However, it has also been shown that Th0-like effector cells secrete significantly more IFN- $\gamma$  when restimulated with B cells, and significantly more IL-4 when restimulated with macrophages (Duncan and Swain, 1994). These findings aroused interest in determining the APC-secreted molecules or surface molecules that may differentially regulate the activity of Th effector cells.

IL-12 produced by macrophages/monocytes not only induces the generation of Th1 cells, it is required, at least *in vitro*, for optimal proliferation and IFN- $\gamma$  production by fully differentiated Th1 cells. While naive T cells require only B7 costimulation for maximal activation, proliferation and IFN- $\gamma$  production by Th1 clones is dependent upon the expression of B7 on accessory cells in synergy with IL-12 (Murphy *et al*, 1994). Keratinocytes preferentially stimulate the production of IL-4 (and not IFN- $\gamma$ ) from human PBMCs, which may relate to their inability to produce IL-12 (Goodman *et al*, 1994). IFN- $\gamma$ , induced by IL-12, has a powerful effect in priming phagocytic cells for production of IL-12. The presence of this positive feedback obviously represents a dangerous trigger for the over-production of inflammatory responses and predicts that a potent negative control exists. The most efficient cytokine for this function is IL-10. The ability of IL-10 to suppress IFN- $\gamma$  production in T cells is primarily due to its inhibition of IL-12 production by APCs (D'Andrea *et al*, 1993; Murphy *et al*, 1994; Kubin *et al*, 1994), and B7 expression on APCs (Kubin *et al*, 1994).

Th1 and Th2 effector cells appear to differ in their dependence on costimulation by APC. It has been shown that murine Th1 clones become anergic when stimulated by antigen receptor ligation in the absence of co-stimulation whereas Th2 clones are activated under the same conditions (Williams *et al*, 1990; Gajewski *et al*, 1994) and that IL-4 secretion by fully differentiated TCR Tg Th2-like cells is not blocked by the B7 antagonist CTLA-4 Ig (McKnight *et al*, 1994). However, while most studies agree that the function of Th1 effector cells is more dependent on costimulation than Th2 effector cells, it has also been shown that CD28 ligation makes Th2 clones more responsive to IL-4 (McArthur and Raulet, 1993). In accordance with this complexity, it has been shown that both IL-4 and IFN- $\gamma$  upregulate B7-2 expression on B cells (Stack *et al*, 1994). Furthermore, there have been reports that Th2 cells can be tolerised (Sloane-Lancaster *et al*, 1994; Garside *et al*, 1995).

Polarized Th responses generated by parasitic infection have also been used as models to study how the growth and function of Th cells with established phenotypes are cross-controlled *in vivo*. Initial evidence for *in vivo* cross-regulation of Th responses came from experiments that showed that CD4<sup>+</sup> Th1 cells can be derived from mice in which a parasite-induced Th2 response exists (Street *et al*, 1990). Powrie and colleagues (1994, 1996) went on to demonstrate the importance of cytokines in the complex regulation of host-protective and pathogenic Th responses to *L. major* infection *in vivo*. CD4<sup>+</sup> cells from non-healing BALB/c mice (4-6 weeks after infection) were separated on the basis of CD45RB expression (Powrie *et al*, 1994). The CD4<sup>+</sup>CD45RB<sup>lo</sup> population contained cells that secreted antigen-specific IL-4 and could transfer a non-healing response to *L. major*-infected SCID mice, while the CD4<sup>+</sup>CD45RB<sup>hi</sup> population contained cells that secreted antigen-specific IFN- $\gamma$  and were capable of transferring protection to *L. major*-infected SCID mice. The transfer of the anti-leishmanial protective Th1 response by the CD45RB<sup>hi</sup> population was inhibited by co-transfer with the CD45RB<sup>lo</sup> population by a mechanism that was dependent on IL-4. In addition, transfer of the CD45RB<sup>hi</sup> population also induced a Th1-mediated colitis in recipient SCIDs that was completely suppressed by co-transfer with the CD45RB<sup>lo</sup> population by a mechanism that was dependent on TGF- $\beta$ , but was independent of IL-4 (Powrie *et al*, 1996). In murine schistosomiasis, IL-10 produced at egg-laying results in the switch from Th1 to a Th2 dominated response (Sher *et al*, 1991). While there has been no direct analysis of the cytokines that are important in sustaining established Th2 responses to nematode infection *in vivo*, IL-4 and IL-10 are predicted to play critical roles, since IL-4 and IL-10 KO mice infected with helminth parasites develop impaired parasite-specific Th2 responses and elevated Th1 responses in comparison to wild-type controls (Kopf *et al*, 1993; Lawrence *et al*, 1995; Kühn *et al*, 1993).

#### 1.4.7. The Stability of Th Polarized Responses. Is There Plasticity in the Th Cell Phenotype?

The success of immunological treatments at the time of infection with various infectious agents, mentioned previously, raised the question of how stable the resultant Th1 or Th2 responses are. While it is clear that such treatments can influence the initial differentiation of Th effector cells from naive cells, they must be given before or at the time of antigenic challenge to be successful and therefore they do not demonstrate that established Th response can be modulated or even switched. Using parasitic and TCR Tg models, various attempts have been made to reverse established Th1 or Th2 responses *in vitro* and *in vivo*.

The transfer of spleen cells, uncloned Th2 cells or IL-4 producing CD45RB<sup>lo</sup> cells from *L. major*-infected BALB/c mice to SCID mice enhanced their susceptibility to subsequent *L. major* infection. However, when the recipient SCID mice were simultaneously treated with anti-IL-4 the animals develop protective Th1 responses (Holaday *et al*, 1991; Powrie *et al*, 1993). While it cannot be ruled out that Th2 cells are switching to Th1 cells, it is more likely that within the leishmanial-specific Th2 population there is a population of Th0 or naive cells that can become Th1 cells. However, it has not been possible to reverse an established leishmania-specific Th1 response transferred to SCID mice using anti-IFN- $\gamma$  *in vivo*.

The opposite results were been obtained from *in vitro* studies. Culture with rIL-4 can convert Th1-like cells derived from *L. major*-infected resistant mice to Th2-like cells (Mocci and Coffman, 1995). This occurs when donor cells are derived from IL-4 KO mice, therefore ruling out the outgrowth of minor Th2 population. In addition, these "switched" cells retained a Th2 phenotype and exacerbated a subsequent *L. major* infection, when transferred into SCID mice. However, the conversion of Th2 cells from *L. major* infected BALB/c mice into Th1-type cells could not be achieved by incubation

with anti-IL-4 and/or IFN- $\gamma$  and IL-12. These results are consistent with the findings of Perez *et al* (1995) using TCR Tg CD4<sup>+</sup> T cells. Fully differentiated Th1 cells can be converted into IL-4 producers by restimulation in the presence of IL-4, whereas the Th2 phenotype is not reversible by exposure to IL-12, even when IL-4 was neutralised.

Therefore, the *in vivo* and *in vitro* data appears to be contradictory. Th1 cells appear to be present in Th2 polarized responses *in vivo*, but not vice-versa, yet a Th1 population can be converted into a Th2 phenotype *in vitro*, but not vice-versa. However, this discrepancy could be due to differences in the *in vivo* versus *in vitro* priming requirements for Th1 and Th2 cells being achieved successfully. Alternatively, the inability to convert from a Th2->Th1 response *in vitro* may reflect the fact that the Th2 pathway is the natural, default pathway of Th cell differentiation *in vivo*. Clearly further experiments are required to appreciate additional factors that may influence the plasticity of established Th responses. For example, since the injection of rIL-12 or anti-IL-4 was unable to reverse the established Th2 response of BALB/c mice that have been infected with *L. major* for three weeks (Chatelain *et al*, 1992; Sypek *et al*, 1993), it was suggested that the high parasite burden in these mice was preventing the conversion to a healing Th1 phenotype. Indeed, treatment with anti-IL-4 (Nabors *et al*, 1994) or IL-12 (Nabors *et al*, 1995) in combination with the leishmanicidal drug Pentostam, induces a switch from a Th2 to a Th1 response and cure of established *L. major* infection in BALB/c mice. Similarly, few studies have reported attempts at switching the established Th2 phenotype of mice infected with nematode infections, which may reflect the difficulty in doing so. However, co-injection of rIL-12 and *B. malayi* mf i.p., after Th2 responses had become established (by immunisation with four weekly s.c. injections of mf antigen) resulted in a significant reduction in antigen-specific IL-4 and IL-5 and increased IFN- $\gamma$  production by spleen and peritoneal cells (Pearlman *et al*, 1995). Despite this, rIL-12 given during a secondary infection with *N. brasiliensis* failed to inhibit the expression of Th2-associated cytokines, although IFN- $\gamma$  expression was stimulated (Finkelman *et al*, 1994).



## 1.5. Immunoregulation II: Apoptosis and Immunity

Apoptosis or programmed cell death (PCD) is a universal property of almost all cells from the most primitive organisms to mammals. The regulation of cell death is necessary for normal development of the animal, for example to ensure the survival of stem cells and the removal of unnecessary or dangerous cells. PCD has also been implicated in defence, homeostasis and ageing and more recently, its inappropriate activation has been blamed for the pathology of many human diseases, including Alzheimer's, stroke, heart attack and AIDS. The term apoptosis was originally coined to describe the morphology of PCD and although the terms are used interchangeably the detection of apoptosis obviously does not elude to either the function or mechanism of PCD. Some cell types undergoing PCD do not display every principal attribute of apoptosis. However, the genetically programmed cascade of cellular events appears to be stereotyped from cell to cell, regardless of the manner in which death is induced (Wyllie *et al*, 1980). The first signs of apoptosis are the condensation of chromatin into large masses around the nuclear envelope. DNA is fragmented by endogenous endonucleases. The cytoplasm then shrinks, membrane blebbing becomes prominent and the nucleus collapses into the densest possible form, although other organelles remain morphologically intact. The cell then fragments into membrane bound bodies that are rapidly engulfed by surrounding phagocytes without releasing their contents. Consequently, apoptosis is rapid, does not evoke an inflammatory response, and usually overtakes single cells rather than groups of cells. In contrast, necrosis, or pathological cell death, is characterised by swelling of the cytoplasm followed by rupturing of the plasma membrane and random DNA degradation by lysosomal DNases. Therefore, PCD, which is also called physiological cell death, is distinguished from necrosis, in that it can occur without damage to surrounding cells and, secondly, it appears to be genetically programmed (Kerr *et al*, 1972).

Lymphocytes appear to be more susceptible to apoptosis than other cell types. This is particularly so during development when lymphocytes that have non-functional, low affinity or autoreactive antigen-receptor rearrangements are deleted by apoptosis, known as central tolerance (Kappler *et al*, 1987; Murphy *et al*, 1990). In the mouse, over 95% of thymocytes and over 70% of pre-B cells are destined to die before they leave the thymus or bone marrow (Egerton *et al*, 1990; Osmond, 1991). Furthermore, physiological death of mature T cells specifically activated by antigens not presented in the thymus would serve as an additional mechanism of tolerance to delete autoreactive cells in the periphery. Equally important, at the end of immune responses, an expanded lymphoid pool is decreased in size by apoptosis to re-establish cellular homeostasis and permit new immune responses to be initiated, a function termed "clonal down-sizing" (reviewed by Sprent, 1994). Apoptosis has also been shown to be involved in peripheral deletion of antigen-reactive cells in oral tolerance (Chen *et al*, 1995; Garside *et al*, 1996), the destruction of damaged lymphocytes (Reed, 1994), and to mediate the cytotoxic action of CTLs (Glass *et al*, 1996) and dendritic cells (Süss and Shortman, 1996) on target cells. Conversely, the apoptotic pathway has been shown to be blocked in mature positively selected thymocytes (Hockenberry *et al*, 1991), in the survival of long-lasting memory lymphocytes (Akbar *et al*, 1993) and in lymphoid malignancy (McDonnell and Korsmeyer, 1991).

Understanding the nature of the biochemical pathways that contribute to apoptosis has been the subject of active investigation for many years. The fact that changes resembling apoptosis can occur in enucleated cells (Jacobsen *et al*, 1994), indicated that the factors initiating apoptosis are to be found in the cytoplasm, and that DNA cleavage, rather than triggering apoptosis, is a means of disposal of DNA from dying cells. A number of cytoplasmic proteins have been discovered that have been termed "survival" and "suicide" proteins, based on their central role in regulating apoptosis of lymphocytes and other cell types. In mammalian cells the most-well characterised are a family of cytoplasmic membrane-bound proteins Bcl-2, Bcl-x<sub>L</sub>, Bax and members of

the ICE (interleukin-1 $\beta$  converting enzyme) family of cytoplasmic cysteine proteases. It has been shown that Bcl-2 and Bcl-x<sub>L</sub> protect cells from death induced by growth factor dependence (Vaux *et al*, 1988; Boise *et al*, 1993), whereas, overexpression of Bax and ICE accelerate cell death (Oltvai *et al*, 1993; Miura *et al*, 1993). Furthermore, co-transfection has demonstrated that Bax can antagonize the protective effect of Bcl-2 (Oltvai *et al*, 1993), while Bcl-2 can block apoptosis promoted by ICE (Boise *et al*, 1993).

Clearly apoptosis is not simply controlled by these few proteins listed above. In fact, the expression of these gene products is under a further level of control. For example, a smaller protein encoded by an alternatively spliced transcript of the bcl-x gene displays the opposite function to the larger version (bcl-x<sub>L</sub>) (Boise *et al*, 1993). As the first physiological inhibitor of cell death to be discovered, bcl-2 has been investigated most thoroughly to determine whether its expression is essential in the development and maintenance of normal cell function (reviewed by Cory, 1995). Initially, it was found that Bcl-2 expression correlated with cellular lifespan in tissues in which it is highly expressed like the nervous system and gut epithelium (Hockenberry *et al*, 1991). In addition, levels of Bcl-2 were found to be high in immature (CD4<sup>-</sup>CD8<sup>-</sup>) thymocytes, as well as in mature single positive (CD4<sup>+</sup>CD8<sup>-</sup> or CD4<sup>-</sup>CD8<sup>+</sup>) T cells, but low in the double positive population of thymocytes that are subject to positive selection. Furthermore, upon activation mature peripheral T cells express lower levels of bcl-2 and become susceptible to apoptosis (Gratiot-Deans *et al*, 1993). Although this would provide a mechanism for clonal down-sizing, it also requires that counter-mechanisms exists to permit the survival of memory cells. Interestingly, it was found that co-culture of activated T cells with fibroblasts permits T cell survival despite low bcl-2 expression (Akbar *et al*, 1993). Therefore, T cell memory may persist *in vivo* as a consequence of a previously primed population receiving continual stimulation, although it is not apparent what the nature of these survival signals are. However, the function of bcl-2 does not appear to be critical during embryogenesis or lymphoid development since bcl-

2 KO mice appear healthy at birth and exhibit normal numbers of lymphoid cells. In contrast, mature T cells from these mice are abnormally susceptible to apoptosis (Veis *et al*, 1993). Consistent with this KO data, over-expression of bcl-2 in transgenic mice does not appear to effect intrathymic clonal deletion, but inhibits SAg-induced peripheral T cell deletion (Sentman *et al*, 1991). While other proteins clearly replace Bcl-2 in bcl-2 KO mice, another important conclusion from both studies is that distinct apoptotic mechanisms act during the deletion of immature and mature T cells, that will be mentioned again later.

A significant advance in our understanding of the multiple pathways that regulate apoptosis was the discovery of surface receptors that signalled for cell death. When T hybridoma cells (Brunner *et al*, 1995; Ju *et al*, 1995), Jurkat T leukemia cells (Dhein *et al*, 1995) and non-transformed, preactivated T cells (Dhein *et al*, 1995) are stimulated via the TCR, they rapidly undergo cell death that is dependent on signalling mediated through Fas/Apo-1/CD95 interacting with its ligand, FasL. This form of apoptosis has been called activation induced cell death, AICD. Following TCR activation both Fas and FasL are upregulated on these cells and death can be inhibited by blocking either Fas or FasL. Cells incubated with a non-stimulatory anti-Fas antibody fragment or a chimaeric Fas-Ig fusion protein do not undergo AICD. Mice carrying a mutation in the Fas (*lpr/lpr*) or FasL gene (*gld/gld*) develop a fatal autoimmune disease characterised in part by the accumulation of large numbers of abnormal T cells (Adachi *et al*, 1993; Watanabe-Fukunga *et al*, 1992; Takahashi *et al*, 1994; Adachi *et al*, 1996) which are relatively resistant to TCR-mediated tolerance and apoptosis (Bossu *et al*, 1993; Ettinger *et al*, 1995; Adachi *et al*, 1996). FasL in *gld* mice carries a point mutation in the C-terminal region, which renders the molecule unable to induce apoptosis in cells expressing Fas (Takahashi *et al*, 1994). The *lpr* mutation is caused by the insertion of an early transposable element (a mouse endogenous retrovirus) into intron 2 of the Fas gene, which causes premature termination and aberrant splicing of the Fas transcript and, therefore, the protein is not expressed. However, a very low level of normally

spliced Fas mRNA or protein is expressed in the thymus indicating that the *lpr* mutation is "leaky" (Adachi *et al*, 1993). Another spontaneous *lpr* mutation, *lpr<sup>cg</sup>*, caused by a point mutation in the Fas gene (isoleucine to asparagine) abolishes the ability of Fas to transduce the apoptotic signal (Watanabe-Fukunga *et al*, 1992). However, to provide conclusive evidence for the role of Fas in the *lpr* disorder, *lpr*-null mice were generated by gene targetting and were shown to develop the same disease as *lpr* mice but at an accelerated rate (Adachi *et al*, 1996). Furthermore, the presence of a soluble form of the Fas molecule (lacking the transmembrane region), that protects from Fas-mediated apoptosis, is elevated in patients with systemic lupus erythematosus, and induces autoimmune features when injected into mice (Cheng *et al*, 1994). Initially it was thought that the pathology in *lpr* mice resulted from defective thymus selection due to lack of Fas expression (Watanabe-Fukunaga *et al*, 1992). However, as described later, it now appears that the selection of T cells in the thymus of *lpr* mice is normal and that it is the failure to delete autoreactive and persistently activated T cells in the periphery that leads to the *lpr* syndrome; the abnormal CD4<sup>+</sup>CD8<sup>-</sup> T cells accumulate because a signal through Fas is required for their removal in the periphery of normal mice.

The restriction of AICD to T lymphocytes is thought to be related to the fact that Fas is upregulated on T cells upon activation. Therefore, apoptosis occurs in activated but not resting peripheral T cells (Wesselborg *et al*, 1993). In addition, the finding that FasL is also transiently expressed on activated T cells (Brunner *et al*, 1995), but is not expressed on any other haemopoietic cells (Suda *et al*, 1995), could provide an additional mechanism, to the competition for survival factors, that prevents over-expansion of an activated T cell population. Furthermore, it has been shown that through their expression of FasL, a population of CD8<sup>+</sup> dendritic cells are functionally specialized to kill activated, Fas-expressing CD4<sup>+</sup> T cells (Süss and Shortman, 1996). It has also been shown that a single T cell can kill itself by Fas-FasL interaction (Dhein *et al*, 1995; Brunner *et al*, 1995), perhaps due to the release of FasL from the surface (Dhein *et al*, 1995), and that cytotoxic CD4<sup>+</sup> T cells expressing FasL, can kill activated

macrophages, that up-regulate Fas, through the Fas pathway (Ashany *et al*, 1995). Therefore, it is not surprising that the expression of FasL on cells, other than activated T cells, is limited. In addition, FasL has been shown to be constitutively expressed in immunoprivileged sites, e.g. the testes and eye, where destructive inflammatory responses cannot be tolerated (Bellgrau 1995, Griffith *et al*, 1995) and on tumours, perhaps as a strategy to escape immune rejection (Hahne *et al*, 1996).

However, it is unlikely that the Fas/FasL interaction is responsible for all forms of apoptosis. Recently, Singer and Abbas (1994) have shown that a major difference between intrathymic and peripheral T cell deletion is the requirement for Fas signalling. These experiments were performed using mice that were bred from a cross between a TCR Tg mouse line (specific for a pigeon cytochrome c peptide) and MRL-lpr/lpr mice or control MRL+/+ mice. Therefore, by generating TCR Tg mice on Fas-deficient (lpr/lpr) and Fas-expressing (+/+) backgrounds, the role of Fas in thymic and peripheral T cell deletion could be assessed to the same antigenic peptide under controlled conditions. *In vivo* administration of the cognate peptide causes deletion of thymic T cells in both MRL-lpr/lpr and MRL+/+ strains. In contrast, antigen-reactive peripheral T cells were deleted in MRL+/+ , but not in the MRL-lpr/lpr strain. Furthermore, while the peripheral clonal deletion of mature T cells against a bacterial SAg was impaired in Fas-null mice, thymic clonal deletion, as assessed by deletion of cells reactive to mouse endogenous SAg, was apparently normal (Adachi *et al*, 1996). Therefore, it appears that Fas expression is required for the death of activated peripheral T cells but not for negative selection of immature thymocytes. While the mechanism of intrathymic clonal deletion is unknown, it has been proposed that thymocytes that fail to express a TCR with sufficient affinity for thymic Class I and II or are unable to productively rearrange their TCR chains may die of "neglect" (Crispe, 1994).

Since the experiments by Singer and Abbas (1994) were carried out in the claustrophobic environment of Tg mice, where virtually all the T cells are specific for

the same peptide, it is not possible to conclude that the Fas pathway is an absolute requirement for the death of all activated peripheral T cells in a normal setting. In addition, although impaired in comparison to wild-type mice, apoptosis can be induced in *lpr* mice, which cannot be attributed to leakiness of the Fas mutation on *lpr* T cells (Bossu *et al*, 1993; Mogil *et al*, 1995; Zheng *et al*, 1995; Van Parjis *et al*, 1996). Furthermore, while self-reactive, abnormal T cells accumulate in *lpr* mice, they respond normally to foreign antigen suggesting that the mechanisms for clonal-downsizing to non-self antigens are intact (Very *et al*, 1993).

In view of these findings, there has been much work done recently to uncover receptors, other than Fas, that are capable of transducing a T cell death signal. It has recently been shown that apoptotic signals delivered through the TCR requires the expression of an orphan steroid receptor, Nur77 (Liu *et al*, 1994; Woronicz *et al*, 1994). However, the focus for most of these studies has been the TNF receptor (TNF-R), signalling through which can also induce apoptosis of mature T cells following stimulation via the TCR (Zheng *et al*, 1995; Bigda *et al*, 1994). p55 and p75 TNF-R and Fas belong to same family of membrane receptors, while FasL is a member of the TNF family (Smith *et al*, 1994). Fas and the p55 TNFR share a cytoplasmic region (the "death box" or Fas-associated death domain, FADD) that is essential for inducing apoptosis via these receptors (Krammer *et al*, 1994). However, p75 TNFR signals via a different intracytoplasmic domain (Smith *et al*, 1994; Bigda *et al*, 1994). Consistent with these findings, it has recently been shown that p75 TNF-R can mediate apoptosis of mature normal lymph node T cells stimulated with anti-CD3 (Zheng *et al*, 1995), while signalling via p55 TNF-R can induce apoptosis of non-lymphoid cells (Schulze-Osthoff *et al*, 1994). The study by Zheng *et al* (1995) was undertaken directly because of the finding that although resting lymph node T cells from *lpr* and *gld* mice exhibited substantially less cell death following cross-linking with anti-CD3 than T cells from wild-type i.e. Fas-positive (C3H) mice, there was still a significant amount of apoptosis. Incubation with anti-TNF serum completely blocked AICD of T cells from *lpr* and *gld*

mice and partially reduced AICD of wild-type T cells. In addition, FasL and TNF induced apoptosis of wild-type T cells displayed different kinetics. At 24h after *in vitro* stimulation with anti-CD3, the AICD observed was mostly due to FasL, but by 48h both FasL and TNF contributed. Furthermore, T cells from p75KO mice (C57BL/6 backgrounds) exhibit less AICD after 48h than T cells from p55KO or wild-type mice. In addition, it was shown that TNF mediates AICD of CD8<sup>+</sup>, but not CD4<sup>+</sup>, T cells from *gld* mice. Therefore, it was concluded that the TNF-p75TNF-R interaction is sufficient for TNF-mediated AICD of mature CD8<sup>+</sup> T cells, but plays a minor role to Fas in AICD of mature CD4<sup>+</sup> T cells. The ability of TNF to mediate Fas-independent mature T cell apoptosis has, therefore, been suggested to account for peripheral deletion that has been detected in *lpr* mice (Bossu *et al*, 1993; Mogil *et al*, 1995; Van Parjis *et al*, 1996). The individual contribution of Fas and TNF-R to T cell apoptosis in normal mice remains to be determined, although their temporal expression following T cell activation, in the above study, reflects distinct roles. In addition, T cell development and activation appears to be normal in p55TNF-R KO and p75TNF-R KO mice, although p55TNF-R KO mice are more susceptible to infection with intracellular pathogens (Erickson *et al*, 1994).

Understanding how and where signalling via Fas and TNF-R interacts with the known cytoplasmic pathway of apoptosis is the subject of much current interest. Despite the ability of Bcl-2 to block apoptosis induced by many other signals (Vaux *et al*, 1988; Oltvai *et al*, 1993; Cory *et al*, 1995), its expression fails to block apoptosis triggered by Fas (Strasser *et al*, 1996) (apart from rare exceptions, e.g. Jurkat cells (Boise *et al*, 1993)) and p55TNF-R (Vanhaesebroeck *et al*, 1993). In addition, there is no evidence that Fas ligation induces expression of Bcl-2 antagonists such as Bax or Bcl-x<sub>s</sub> (Strasser *et al*, 1995), suggesting that either there is more than one biochemical mechanism of apoptosis, or more likely, there are certain effectors that operate downstream of Bcl-2. There is increasing evidence that apoptosis triggered via Fas or p55TNFR depends on ICE or another member of that family. CrmA, a specific inhibitor of ICE (Nicholson *et*



*al*, 1995), enhanced the survival of several lymphoid lines treated with anti-Fas antibody (Los *et al*, 1995) or TNF (Enari *et al*, 1995). Therefore, it could be postulated that ICE and its close relatives participate at a point in the apoptotic pathway downstream from the step inhibitable by Bcl-2. Some further advances have been made in understanding the contribution of the receptors themselves. It is thought that aggregation of Fas, following FasL interaction, results in association with FADD and other proteins that is then able to activate ICE-like proteases (Enari *et al*, 1995; Los *et al*, 1995) that in turn cleaves and inactivates poly(ADP-ribose) polymerase (PARP), an enzyme used for DNA repair (Nicholson *et al*, 1995). Although cleavage of PARP itself appears to be dispensible for apoptosis, it can be used as a convenient marker of the apoptotic cascade resulting in DNA fragmentation (Nicholson *et al*, 1995).

The relationship between cell death and anergy is also poorly understood. Although most studies indicate that Fas mediates a death signal, there is some evidence that Fas ligation can act as a costimulatory signal (Alderson *et al*, 1993). It has also been shown that CD28 costimulation, while having no effect on bcl-2 expression, does upregulate bcl-x<sub>L</sub> and that this upregulation correlates with protection from cell death by a Fas-dependent mechanism (Boise *et al*, 1995). Furthermore, signalling via CTLA-4, that is also known to inhibit T cell activation, can induce apoptosis (Gribben *et al*, 1995). A recent study used TCR Tg mice, on Fas-expressing or Fas-deficient backgrounds, to assess more directly the role of costimulation and Fas in the induction T cell apoptosis and anergy (Van Parijs *et al*, 1996). Based on these experiments it appears that apoptosis of naive cells, due to culture in the absence of antigen, is not mediated by Fas, but could be prevented by CD28-mediated signals that upregulate bcl-x<sub>L</sub>. In contrast, activated T cells that undergo apoptosis as a result of repeated stimulation (AICD) is dependent on Fas but is independent of costimulation or the expression of bcl-x<sub>L</sub>. Therefore, it has been suggested that apoptosis as a result of inadequate stimulation should be referred to as PCD to distinguish it from AICD. In addition, in this model peripheral tolerance could be induced *in vivo* in Fas-deficient mice.

Apoptosis can also be regulated by cytokines. As mentioned above, IL-2 was shown to augment Fas-mediated AICD (Lenardo, 1991; Van Parijs *et al*, 1996), possibly by inducing a larger proportion of cells to progress to a specific susceptible point in the cell cycle (Lenardo, 1991). It is thought that mature T cells that have progressed to S phase are susceptible to receptor-mediated apoptosis, while T cells (blocked) in G1 phase are refractory (Boehme and Lenardo, 1993). Therefore, in this context it is not surprising that growth factors like IL-2, that drive T cells into cycle, promote apoptotic cell death of T cells after their subsequent activation with SEB (Lenardo, 1991) or high dose of antigen (Critchfield *et al*, 1994). Triggering of T cell apoptosis, under conditions of high antigen load and IL-2, could, therefore, contribute to the mechanism preventing unrestricted proliferation of T cells. As a corollary to this, proliferation has been shown to be a prerequisite for SEB induced T cell apoptosis (Renno *et al*, 1995; Mietke *et al*, 1996). Furthermore, mice in which the gene for IL-2 or IL-2 receptor  $\beta$  chain have been deleted develop autoimmunity and are resistant to AICD (Sadlack *et al*, 1995; Susuki *et al*, 1995). IFN- $\gamma$  appears to be directly involved in cell death induced by *in vitro* stimulation of thymocytes, activated human T cells or a murine Th1 clone with anti-CD3 in the absence of accessory cells (Liu and Janeway, 1990; Groux *et al*, 1993).

Induction of apoptosis in mature T cells has been demonstrated during infection with intracellular pathogens (Lopes *et al*, 1995; Razvi and Welsh, 1993; Akbar *et al*, 1993; Rogers *et al*, 1996). It has been suggested that apoptosis of mature T cells could play a role in immunosuppression caused by viral, bacterial and protozoal infection, although it is not clear whether this functions solely to promote survival of the foreign organism or whether it reflects a dysregulated host-protective mechanism to remove infected cells. As yet the mechanisms of apoptosis have not been defined in these models.

## **CHAPTER 2**

## **Chapter 2 Materials and Methods**

### **2.1. The parasite**

#### **2.1.1. Maintenance of the mosquito life cycle**

*Aedes aegypti* (ref<sup>m</sup>) mosquitoes were kept in cages in a purpose-built insectary at a temperature of 28°C and a relative humidity of 75-80%. To maintain stocks, mosquitoes were fed twice weekly with heparinized bovine blood using an artificial membrane-feeder. Moist Whatman 3MM filter papers were placed in the cages to collect mosquito eggs and then removed and dried until use. Eggs were hatched by transferring the filter papers to plastic trays of tap water containing yeast tablets to feed the developing larvae. Pupae were picked daily and transferred to cages. Adult mosquitoes were fed on sucrose and starved for 24h prior to receiving a blood-meal.

#### **2.1.2. Maintenance of the parasite life cycle**

Adult parasites and microfilariae (mf) were obtained from the peritoneal cavity of jirds (*Meriones unguiculatus*) that had been infected for at least three months with 250 *B. pahangi* L3. Jirds were killed by CO<sub>2</sub> inhalation and exsanguinated by cardiac puncture. Adult worms were washed in Hank's Balanced Salt Solution (HBSS) and frozen in liquid nitrogen to be used as a source of parasite material for extracts (see 2.1.4). The mf were washed in HBSS and resuspended in heparinized rabbit blood at a concentration of approximately 400/20µl. Mosquitoes were fed using the artificial membrane feeder.

#### **2.1.3. Recovery of infective larvae and microfilariae for experimental infections**

L3 were harvested from infected mosquitoes at day 9 p.i. using standard methods (Devaney and Jecock, 1991), washed extensively in sterile HBSS and counted. The Mf were obtained from the peritoneal cavity of infected jirds by extensive washing in

HBSS and separated from host cells by centrifugation over Histopaque-1077 (Sigma) at 1,500 rpm for 15 mins without brake. Mf were then washed twice in HBSS (1,000 rpm, 5 mins), resuspended and counted.

#### **2.1.4. Preparation of adult antigen extracts**

Soluble extract of *B. pahangi* adult worms, for use in cell culture experiments, was prepared by homogenisation in RPMI (1640 Dutch Modification, containing 5mM glutamine, 5mM HEPES, 100U/ml penicillin and 100µg/ml streptomycin. All from Gibco) on ice. The homogenate was incubated on ice for a further 60 min with occasional mixing. The suspension was centrifuged at 10,000g for 30 min at 4°C. The supernatant was sterilised by filtration through a 0.45µm Spin-X filter unit (Costar), assayed for protein concentration using the BioRad method and stored at -70°C until use.

DOC (Sodium deoxycholate) extract of *B. pahangi* adult worms, for use as the target antigen in antibody subclass ELISA, was prepared by homogenisation on ice in 10mM Tris-HCl pH 8.3, containing the protease inhibitors: 20mM phenylmethylsulphonylfluoride, 2mM L-1-tosylamido-2-phenyl-ethylchloromethylketone and 2mM N-α-pTosyl-L-lysine chloromethylketone. DOC was added to give a final concentration of 1% and the homogenate was incubated on ice for 30 min with occasional mixing. The suspension was centrifuged at 10,000g for 10 min at 4°C. The supernatant was assayed for protein concentration using the BioRad method and stored at -70°C until use.

## **2.2. Analysis of the primary immune response to L3 and mf**

### **2.2.1. Animals and infection protocols**

6 week old male BALB/c were purchased from Harlan-Olac (Bicester, U.K.) and were maintained in filter-topped cages. Groups of 5 BALB/c were injected s.c. with either 50

L3,  $1 \times 10^4$  or  $1 \times 10^5$  mf, or an equivalent volume of HBSS in "the scruff of the neck". Heat-killed L3 were prepared by plunging into a boiling water bath for 2 min. At day 12 following infection, mice were killed by CO<sub>2</sub> inhalation. Spleens were removed and serum collected by cardiac puncture. In additional experiments, groups of 6 week old male C57BL/6 mice (also purchased from Harlan-Olac) and 7 week old MRL-lpr/lpr and MRL-lpr/C57BL/6 mice (obtained from Dr E. Cameron, University of Glasgow) were infected with either 50 L3,  $1 \times 10^5$  mf or HBSS using the same protocol as that described for BALB/c mice.

### **2.2.3. Preparation of spleen cells**

Spleens were washed in RPMI and teased apart to a single cell suspension using sterile needles and syringes. Erythrocytes were lysed by treatment with 0.83% NH<sub>4</sub>Cl (pH 7.2) (See 2.6), washed twice in RPMI and the number of viable lymphocytes assessed by trypan blue exclusion. The cells were resuspended at  $1 \times 10^7$ /ml (proliferation) or  $2 \times 10^7$ /ml (cytokines) in RPMI supplemented with 20% heat-inactivated FCS (Australian, Gibco), to give a final concentration of 10%.

### **2.2.4. Proliferation Assay**

Spleen cell proliferation was measured by the incorporation of <sup>3</sup>H thymidine. Triplicate 200µl cultures ( $1 \times 10^6$  cells/well) in 96-well flat bottomed plates (CoStar) were incubated in the presence or absence of 1µg/ml ConA (Sigma) or 10µg/ml adult antigen (see 2.1.4). The concentrations of ConA and adult antigen were determined to be optimal for proliferation in preliminary experiments. After 48h (ConA) or 72h and/or 96h (antigen) of culture at 37°C and 5% CO<sub>2</sub>, the cells were pulsed for 16h with 0.5µCi of <sup>3</sup>H thymidine/well (1mCi/mmol, Amersham). The cells were harvested and radioactivity measured in a "TopCount" Microplate Scintillation Counter (Canberra Packard Instrument Company).

### **2.2.5. Analysis of IL-2, IFN- $\gamma$ , IL-4, IL-5 and IL-10 production by ELISA**

Spleen cells were incubated at  $1 \times 10^7$  cells/ml in duplicate 1ml cultures in 24-well flat bottomed plates (CoStar) in the presence of ConA (5 $\mu$ g/ml) or antigen (10 $\mu$ g/ml) and the supernatants harvested after 48h and stored in aliquots at -70°C. Levels of IL-2, IFN- $\gamma$ , IL-4, IL-5 and IL-10 were measured by two-site ELISA using antibody pairs purchased from PharMingen. The optimal concentrations for the capture and detecting antibodies were determined in preliminary experiments. Results are expressed as U/ml by reference to commercially produced standards of rIL-2 (Sigma), rIL-4, rIL-5, rIFN- $\gamma$  (PharMingen) or rIL-10 (Genzyme). The limit of detection for each assay was defined as the mean + 3 SD of 16 wells containing medium (RPMI/10%FCS) only.

The cytokine ELISA protocol was performed as follows. ELISA plates (Corning Easy-Wash) were coated with capture antibody in PBS, 50 $\mu$ l/well, over-night at 4°C and then blocked with 10% FCS in PBS/0.05% Tween 20 (See 2.6), 150 $\mu$ l/well, for 45 min at 37°C. During this incubation, samples were defrosted at R.T. and doubling dilutions of the recombinant cytokine standards were prepared in RPMI/10%FCS on a separate plate. ELISA plates were washed as before. Samples, standards and RPMI/10%FCS were transferred to the ELISA plates, 50 $\mu$ l/well, for 2h at R.T. The plates were washed as before and biotinylated antibody diluted in 1% BSA in PBS/0.05% Tween 20 was applied at 50 $\mu$ l/well, for 1h at R.T. The plates were washed as before. Streptavidin peroxidase (Serotec) diluted 1/1000 in 1% BSA in PBS/0.05% Tween 20 was applied at 75 $\mu$ l/well, for 1h at R.T. The plates were washed as before. TMB peroxidase substrate (KPL) was added at 100 $\mu$ l/well, for 15 mins and then the plates were read at 620nm in a Dynatech MR5000 automated ELISA reader.

### **2.2.6. *in vitro* treatments**

In some experiments rat anti-mouse IL-4 MAb (11B11, supplied by the Biological Response Modifiers Program, National Cancer Institute), rat anti-mouse IL-10 MAb (JESS, a kind gift from Dr. R. Grencis, University of Manchester) or an IgG1 isotype

matched control MAb (R59-40, PharMingen) were added simultaneously with ConA or antigen to the spleen cell cultures, at a final concentration of 10µg/ml. Similarly rIL-2 (a kind gift from Dr. C. Lawrence) was added at a final concentration of 100 U/ml. After such treatment the proliferation and cytokine responses of the cells were assessed as described before. IL-4 and IL-10 were assayed for and not detected in any 11B11-, or JESS-treated cultures, respectively, demonstrating that the cytokines had been successfully neutralised.

In certain experiments, resident APCs were depleted from spleens of infected mice by passing the spleen cell suspension over a nylon wool column. Columns were prepared by tightly packing 10ml syringes to the 7 ml mark with pre-scrubbed nylon wool (Leuko-pak, Fenwal Laboratories), followed by autoclaving. The columns were rehydrated by running through 50mls of RPMI supplemented with 20% FCS. A further 10mls of RPMI/20% FCS, was added to the columns which were then sealed with Nescofilm and a 25G needle and placed in a 37°C incubator for at least 90 min before use. A maximum of  $1 \times 10^8$  spleen cells suspended in RPMI/10% FCS were added dropwise to each column which was then re-sealed and placed back in a 37°C incubator for 45min. After incubation, purified T cells were eluted by adding 20mls of RPMI/10% FCS to each column and collected in a 50ml centrifuge tube. The cells were washed (1,000rpm for 5 min), resuspended and counted. Two sources of APCs were prepared by irradiating spleen cells from uninfected or L3 infected mice;  $1 \times 10^7$  spleen cells/ml in RPMI/10% FCS were irradiated at 2500 rads ( $^{137}\text{Caesium}$  source, Department of Veterinary Physiology, University of Glasgow). Following irradiation, the APCs were centrifuged (1000rpm for 5 min) and resuspended in fresh RPMI/10% FCS. The nylon wool non-adherent (T) cell population and irradiated APC populations were combined at various ratios, as detailed in Expts. 4.2.5-8, and proliferation and cytokine production assessed, as described above.



### **2.2.7. Preparation of immune serum**

Blood was collected by cardiac puncture into 1ml syringes and left to clot over-night at 4°C. Serum was then pipetted into screw-top, sterile Eppendorfs and centrifuged at 10,000 rpm for 10 min at 4°C. The serum was then aliquoted and stored at -20°C until use.

### **2.2.8. Antibody isotype ELISA**

Levels of parasite specific IgG, IgG1, IgG2a and IgE in serum samples from individual mice were measured by ELISA using standard methods. Briefly, 96 well flat bottomed Immunolon IV plates (Dynatech) were coated overnight at 4°C with DOC adult extract (see 2.1.4) in carbonate buffer (See 2.6) at 1µg/ml (IgG, IgG1 and IgG2a) or 5µg/ml (IgE). Serum from individual mice was applied in duplicate at dilutions of 1/25 (IgE), 1/50 (IgG2a), 1/100 (IgG1) or 1/200 (IgG) in PBS/0.05% Tween 20. Plates were washed three times with PBS/0.05% Tween 20 between each layer. HRPO conjugated goat anti-mouse IgG (Serotec, 1/9000), anti-mouse IgG2a (Southern Biotechnology Associates Inc.(SBA), 1/2000), anti-mouse IgG1 (SBA, 1/1000) or anti-mouse IgE (Nordic Immunological Laboratories, 1/6000) were used as detecting antibodies diluted in PBS/0.05% Tween 20. After addition of TMB substrate (KPL, Dynex) the specific antibody levels were measured in a Dynatech MR5000 automated ELISA reader and the values expressed as OD 620nm readings.

## **2.3. Analysis of early cytokine mRNA expression following infection with L3 and mf.**

### **2.3.1. Animals/Infection Protocol**

In these experiments, groups of 5-10, male BALB/c mice (Harlan-Olac), maintained in filter-topped cages, were injected, at 6 weeks of age, with 50 L3,  $6 \times 10^4$  mf or HBSS into each hind footpad. Mice were killed by CO<sub>2</sub> inhalation at 24h, 4d or 7d p.i. and the popLNs removed. In one experiment, groups of 3-5, male BALB/c and C57BL/6 mice,

at 6 weeks of age, were injected i.v. with either 50 L3 or HBSS and mice were killed at 2h, 6h or 24h p.i. and spleens removed. Serum was not collected in these experiments.

### **2.3.2. RNA extraction**

At specified time points after infection (24h, 4d and 7d) via the footpad the draining popliteal lymph nodes (popLN) were removed from BALB/c mice, frozen immediately in liquid nitrogen and ground to a fine powder. Alternatively, at specified time points after i.v. injection (2h, 6h, 24h) spleens were removed from BALB/c and C57BL/6 mice, teased to a single cell suspension, as described previously. Following red blood cells lysis, the spleen cell suspensions were pelleted, frozen in liquid nitrogen and ground to a fine powder. Total RNA was extracted from popLN and spleen preparations using Trizol (Gibco), according to the manufacturer's instructions, DNase-treated (DNase I amplification grade kit; Gibco) to remove contaminating DNA and quantified by the intensity of ethidium bromide staining on 1.2 % agarose mini-gels. RNA was stored in DEPC-treated H<sub>2</sub>O (See 2.6) at -70°C until use.

Before the gel was poured, the mini-gel tank and combs were rinsed once in a 0.25M HCl solution, and three times in DEPC H<sub>2</sub>O. For a 50 ml mini-gel, 0.6g agarose was melted in a solution containing 5mls of 10X MOPS buffer (See 2.6) and 36.5 mls of DEPC H<sub>2</sub>O by boiling in a microwave. The solution was cooled to 55°C. In a fumehood, 8.5mls of 17% formaldehyde was then added, mixed and the gel was poured. To 1-5µl of each RNA sample was added 1µl of 500ng/ml ethidium bromide (See 2.6), 1µl loading buffer (See 2.6) and 1µl of formamide. Prior to electrophoresis, the RNA samples were denatured by heating to 65°C for 10 mins. When the gel was set, the RNA samples were loaded and the gel was run in 1xMOPS buffer at 40V for 30-45 mins.

### 2.3.3. Cell selections for RNA

In the selection experiments, the popLN were removed from BALB/c mice at 24 h p.i. with L3, teased to a single cell suspension and incubated with Mab against CD4 (RMA-5, Pharmingen), CD8 (53-5.8, Pharmingen), CD3 (145-2C11, Pharmingen), Thy1.2 (53-2.1 Pharmingen),  $\alpha\beta$  TCR (H57-597, Pharmingen) or  $\gamma\delta$  TCR (GL3, kindly provided by Dr. R. Grencis, University of Manchester). At day 4 p.i. pop LN were removed from L3 infected BALB/c mice and the resulting cell suspension incubated with anti-CD4 MAb. Cells were selected using the appropriate secondary Ab coupled to magnetic particles followed by three rounds of exposure to a magnetic field (Advanced Magnetics, Inc., Cambridge, MA). During magnetic cell sorting cells were kept at 4°C to ensure mRNA stability. RNA extraction was performed on the separated populations as before. The purity of the designated selections exceeded 85-100% as assessed by FACS analysis (Epics Elite, Coulter). In these experiments, RNA was also made from unseparated popLN cells removed from uninfected animals at the same time point post-injection with HBSS. Similarly, in each experiment a small number (a minimum of  $1 \times 10^7$ ) of popLN cells from L3-infected animals was kept unseparated for RNA preparation.

T cells, from popLN of BALB/c mice at 24h p.i. with L3, were also positively selected using murine T cell enrichment mini-columns from R&D Systems. According to the manufacturer's instructions, when  $2 \times 10^8$  cells are loaded onto the mini-columns, the total T cell recovery would be approximately 60% and T cell purity (numbers of CD3<sup>+</sup> cells, as assessed by FACS) would be 87%. However, if less than  $5 \times 10^7$  cells are loaded initially the T cell recovery is dramatically reduced. In a preliminary experiment (data not shown),  $1.2 \times 10^8$  spleen cells (78.0% CD3<sup>+</sup>, as assessed by FACS) were loaded onto one column. Although,  $7.3 \times 10^7$  cells were eluted (60.8% recovery), indicating that some selection had occurred, the population was only fractionally more pure (79.5% CD3<sup>+</sup> as assessed by FACS) than the starting population. Therefore, in an attempt to increase the purity of the T cell population recovered, two mini-columns

were used, so that the eluted population from the first column was purified on a second column. In the experiment shown in 5.2.3,  $4.6 \times 10^7$  pooled popLN cells (70.2% CD3<sup>+</sup> as assessed by FACS) from mice, 24h after infection with L3, were applied to the first column. Although the starting cell number was below the optimum,  $2.85 \times 10^7$  cells were recovered, representing a 69% recovery. However, the population was only 80% CD3<sup>+</sup>. When this eluted population was loaded onto the second column, only  $9.8 \times 10^6$  cells were obtained (34% recovery), but the population was 95.5% CD3<sup>+</sup> (as assessed by FACS). RT-PCR was then performed on RNA prepared from this enriched T cell population and unseparated popLN cells from both L3-infected and uninfected mice.

#### **2.3.4. FACS staining**

Cells to be stained were washed three times (1,000rpm for 5 min) in DAB-2-Azide (See 2.6) and resuspended to  $5 \times 10^6$  cells/ml. A 200 $\mu$ l aliquot ( $1 \times 10^6$  cells) was placed in each FACS tube (Falcon). The cells were spun to a pellet (1,000rpm for 5 min) and resuspended in 100 $\mu$ l of primary antibody at a 1:500 dilution in DAB-2-Azide on ice for 30 min. The primary antibody was either a FITC-conjugate (anti-CD4 YTS 191.1.2, Sera-Lab; anti-CD8 YTS 169.4, Sera-Lab; anti-rat IgG2b isotype matched control NORIG 7.16.2, Sera-Lab; anti-CD3 145-2C11, PharMingen) or unconjugated anti- $\gamma\delta$  (GL3, Dr. R. Grencis, University of Manchester) or anti- $\alpha\beta$  (H57-597, PharMingen) Mabs. Following  $\gamma\delta$  and  $\alpha\beta$  staining, cells were washed and incubated with FITC-anti-hamster IgG (G192-1, PharMingen) at a 1:500 dilution in DAB-2-Azide on ice for 30 min. Since no hamster isotype matched controls were available, fluorescence of cells stained with secondary antibody alone was used as a control for positive staining for  $\gamma\delta$  and  $\alpha\beta$ . In the final step stained cells were washed three times (1,000rpm for 5 min) in 300 $\mu$ l of DAB-2-Azide and then resuspended in 200 $\mu$ l of DAB-2-Azide plus 200 $\mu$ l of 2% formaldehyde in PBS. Stained cells were stored at 4°C in the dark and then analyzed for FITC fluorescence at an excitation wavelength of 495nm on a Coulter flow cytometer.

### 2.3.5. RT-PCR detection of IL-2, IFN- $\gamma$ , IL-4 and IL-10 mRNA

The reverse transcription reaction was carried out in a total volume of 20 $\mu$ l. 2 $\mu$ l (1 $\mu$ g) of random hexamer primers (Promega) and 1 $\mu$ l of RNAsin (Promega) were added to 11 $\mu$ l of total RNA (1 $\mu$ g) and the mix was heated to 65°C for 5 min and then cooled on ice for 5 min. 5 $\mu$ l of Reverse Transcriptase (RT) buffer (Promega), 2.5 $\mu$ l of 10mM dNTP mix (Perkin Elmer), 2.5 $\mu$ l of double distilled, sterile H<sub>2</sub>O and 1 $\mu$ l of murine Moloney leukemia virus RT (Promega) were added and incubated at 42°C for 45 min. Finally the reaction was heated to 90°C for 5 min to denature the RT enzyme, chilled on ice and stored at -20°C.

The PCR used gene specific primers for IL-2, IFN- $\gamma$ , IL-4, IL-5, IL-10 and  $\beta$ -actin (Clontech) that were additionally designed to discriminate between product amplified from cDNA and that amplified from contaminating genomic DNA. To 4 $\mu$ l of RT mix were added:- 1) 5 $\mu$ l 10x PCR buffer (Perkin Elmer), 2) 3 $\mu$ l of 10mM dNTP mix (Perkin Elmer), 3) 1 $\mu$ l of 20mM sense primer, 4) 1 $\mu$ l of 20mM anti-sense primer, 5) 35.5 $\mu$ l of double distilled, sterile H<sub>2</sub>O and 6) 0.5  $\mu$ l (5 U/ $\mu$ l) of Amplitaq (Perkin Elmer). cDNA prepared from spleen RNA was diluted 1/20 before use. The cycling conditions were 94°C for 45 s, 60°C for 45 s, 72°C for 2 min and a final extension of 72°C for 10 min. For each primer pair, the optimal number of cycles was determined experimentally such that a linear relationship between input RNA and final PCR product was obtained. The number of PCR cycles selected for each gene were as follows: IL-2 (20), IFN- $\gamma$  (25), IL-4 (25), IL-10 (20) and  $\beta$ -actin (15).

10 $\mu$ l of the final PCR reaction was run on a 1% agarose gel in 1xTAE buffer (See 2.6) at 50V for 3hrs, Southern blotted and hybridised using the <sup>32</sup>P-labelled PCR product as a specific probe as described in Svetic *et al* (1991). Briefly, after electrophoresis was complete, the gel was placed in denaturation buffer (See 2.6) for 45 min with constant agitation. The gel was rinsed briefly in ddH<sub>2</sub>O and soaked in neutralisation buffer (See 2.6) for 30 min with constant agitation. PCR products were then transferred to nylon

membranes (Hybond-N, Amersham) using 20xSSC (See 2.6) by standard Southern blotting methods. Successful transfer was confirmed by restaining the gel with Ethidium bromide (See 2.6). After transfer, the damp membranes were treated in a Stratagene UV Cross-Linker (150 mJoules). Blots were incubated at 42°C for 6h in pre-hybridisation buffer (See 2.6). Pre-hybridisation buffer was then replaced for 16h at 49°C with hybridisation buffer (See 2.6) containing the appropriate PCR product random-primed with  $^{32}\text{P}$ -dCTP (Amersham) using the High Prime labelling system (Boehringer-Mannheim). Before addition to the hybridisation solution, the labelled probe was separated from unincorporated  $^{32}\text{P}$ -dCTP using Nick-columns (Pharmacia). After hybridisation, blots were washed for 15 min in 6xSSPE (See 2.6), 0.1% SDS, and then 3-4 min in 1xSSPE, 0.1% SDS at 49°C. Autoradiographs were exposed at -70°C using Kodak film (Amersham) and the band intensities quantified by laser densitometry (Molecular Dynamics).

### **2.3.6. Analysis of cytokine secretion by CD4<sup>+</sup> popLN T cells**

In these experiments mice injected with 30 L3 or HBSS were killed at day 10 p.i. and the popLNs draining the infected footpads were removed aseptically. Single cell suspensions were prepared by forcing the nodes through fine nylon mesh into RPMI. The number of viable cells was assessed by trypan blue exclusion. CD4<sup>+</sup> cells were separated from the total popLN population by positive selection using the miniMACS system according to the manufacturer's instructions (Miltenyi Biotec). The efficiency of separation was determined by FACS analysis at >95% (as described in 2.3.4). The purified CD4<sup>+</sup> cells were then plated out in duplicate 1ml cultures at  $4 \times 10^6$ /ml in the presence of  $6 \times 10^6$  irradiated syngeneic spleen cells/ml with antigen (10µg/ml). PopLN cells were irradiated as described for spleen cells in 2.2.6. Unseparated popLN cells from L3 infected mice and uninfected controls were incubated at  $1 \times 10^7$ /ml in the presence of antigen (10µg/ml). Culture supernatants were harvested after 48h and the levels of IL-2, IFN-γ, IL-4, IL-5 and IL-10 were measured by specific two-site ELISA (as described in 2.2.5).

## **2.4. Detection of apoptotic spleen cells in mice infected with mf**

### **2.4.1. Animals/Infection Protocol**

6 week old male BALB/c mice were injected with either 50 L3,  $1 \times 10^3$ ,  $1 \times 10^4$ ,  $1 \times 10^5$ ,  $2 \times 10^5$  mf or HBSS. MRL-lpr/lpr and MRL-lpr/C57BL/6 mice were injected with  $1 \times 10^5$  mf or HBSS. Mice were killed at day 12 p.i. by CO<sub>2</sub> inhalation. Spleens were removed and either teased to single cell suspension and red blood cells lysed, as described in 2.2.3, or placed whole into 4% buffered neutral formalin (BNF).

### **2.4.2. Cell viability assessed by trypan blue exclusion**

Spleen cell suspensions were prepared for culture as described in 2.2.3. Duplicate 200µl cultures were prepared as described in 2.2.4, at  $1 \times 10^6$  cells/well in the presence of medium or adult antigen (10µg/ml). The number of viable cells remaining in culture at 24, 96 and 120h was assessed by trypan blue exclusion.

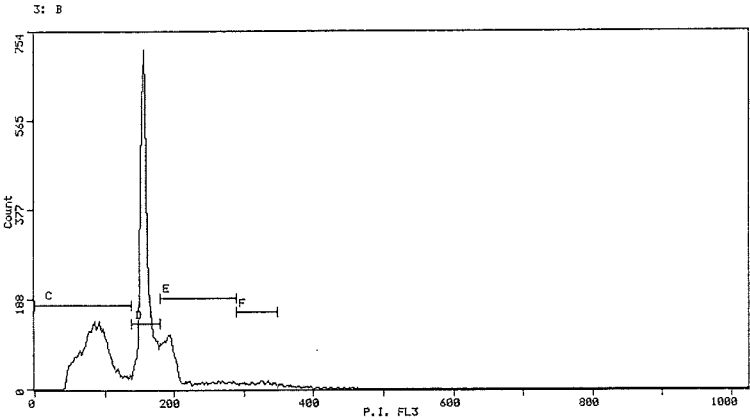
### **2.4.3. Propidium Iodide Staining/FACS analysis**

Nuclear DNA content was assessed by flow cytometric analysis of propidium iodide (PI, Sigma) stained cells as described in Vindelov *et al* (1983). Briefly,  $1 \times 10^6$  cells (fresh or from a 200µl culture well, see 2.4.2), re-suspended in 200µl of PBS were placed in a FACS tube (Röhren) and incubated for 10 min at R.T. with 450µl of 0.03 mg/ml trypsin (Sigma) in stock buffer (See 2.6). The solution was inverted occasionally to mix. To this was added 250µl of a solution containing 0.5 mg/ml trypsin inhibitor (Sigma) and 0.1 mg/ml ribonuclease A (Sigma) in stock buffer and the mixture was incubated at R.T. for 10 min. Again the solution was inverted occasionally to mix. Finally, 250µl of a solution of 0.416 g/ml PI and 1mg/ml spermine tetrahydrochloride in stock buffer was added and the mixture incubated in the dark on ice for a further 10 mins. Stained cells were analysed for PI fluorescence at an excitation wavelength of 488nm on a Coulter EPICS XL flow cytometer (Coulter), to determine the relative amount DNA/cell. Cells with hypodiploid content of DNA (i.e. less DNA than cells in

G1 phase) were considered to be apoptotic (APOP). Cell with double the amount of G1 DNA are in either G2 or M phase, while cells in S phase have intermediate amounts. The DNA analysis program allows these gates to be set for each sample, based on calibration with chicken erythrocytes that possess half DNA content of normal mouse cells. A sample read-out is given below.

UNIVERSITY DEPARTMENT OF SURGERY, WI, GLASGOW.  
COULTER(R) EPICS(R) HISTOGRAM DISPLAY

05Jul96 09:32:34  
DNA ANALYSIS  
ja018  
#00019711



SAMPLE INFO										
PROTOCOL	:	DNA ANALYSIS			HIST COUNT	:	20,960	LIST FILE	:	00019711.LHD
DATA RATE	:	Unknown						PROT FILE	:	
INSTRUMENT	:	XL V44262						SAMPLE DATE	:	05Jul96
SAMPLE NAME	:	ja018						SAMPLE TIME	:	09:32:34
SAMPLE NUMBER	:	00019711								
COMMENTS	:									
STATISTICS										
SINGLE PARAMETER STATISTICS										
.....Peak.....      .....X Channel.....										
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	34.3	7191	83	153	86.7	21.2	24.4	19.2	0	139
D	45.0	9424	157	754	158.8	7.6	4.78	2.57	139	179
E	16.8	3523	192	121	207.5	29.5	14.2	12.7	179	288
F	3.1	653	326	20	317.8	16.7	5.27	0.886	288	348
C Low Channel = 0, High Channel = 139    apo										
D Low Channel = 139, High Channel = 179    G0										
E Low Channel = 179, High Channel = 288    S PHASE										
F Low Channel = 288, High Channel = 348    G2M										

#### 2.4.4. DNA fragmentation

The DNA fragmentation method for determination of apoptosis on 2% agarose gels was based on the technique of Dr. Sarah Howie in the Department of Pathology, University of Edinburgh. A total of  $2 \times 10^6$  cells (fresh or from a 200µl culture well, see 2.4.2) were pipetted into 1ml Eppendorfs, centrifuged and washed in 300µl of PBS to remove FCS. Cells were pelleted again, snap frozen in liquid nitrogen and stored at -70°C until use.



Each pellet was incubated at 50°C for 2h in 20µl of 0.5mg/ml proteinase K in buffer (See 2.6). To each Eppendorf was added 10µl of 0.5mg/ml DNase free RNase in buffer (See 2.6) for 1h at 50°C. At this point samples could be snap frozen in liquid nitrogen and stored at -70°C until required. Gilson pipette tips and loading buffer (See 2.6) were pre-warmed to 70°C before use. Immediately before loading, 10µl of melted loading buffer was added to each tube and the samples loaded into dry wells of a 2% agarose gel containing 1µl of 10µg/ml Ethidium bromide per 100ml 1xTBE running buffer (See 2.6). Samples were allowed to "set" for 5 min before 1xTBE running buffer and markers were added. The gel was run for 2-3h at 75V and visualised under U.V. The sensitivity of this assay has been calculated to be approximately 15% i.e.  $3 \times 10^5$  apoptotic cells/ $2 \times 10^6$  total cells.

#### **2.4.5. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labelling (TUNEL)**

The TUNEL staining technique described here was adapted from the method of Gavrieli *et al* (1992) by Dr Karen Blythe and colleagues in the Department of Veterinary Pathology, University of Glasgow. Briefly, whole spleens were fixed in NBF and embedded in paraffin wax by Mr I. McMillan and Lab 5 staff (Department of Veterinary Pathology, University of Glasgow). 1-2µm sections were adhered to slides and dewaxed at the time of use by incubation in Histoclear (xylene substitute) for 15 mins. Hydration was then performed by washing the slides twice in absolute alcohol for 1min, once in 95% methylated spirit for 2min, and then tap water. Slides were then rinsed and held in dd H<sub>2</sub>O.

Two slides of the same section were processed. The test section was treated with all incubations. The other slide treated with all incubations, except the TdT enzyme/dUTP-biotin step, served as the negative control. As a positive control at least two additional sections were pre-treated with 10 µg/ml DNase (Boehringer Mannheim) in a solution (See 2.6) for 20 min at R.T. and then washed 4x1 min in PBS.

All slides were then covered with 20 µg/ml proteinase K (Boehringer Mannheim) in ddH<sub>2</sub>O for 20 min at R.T., to strip proteins from the nuclei of the sections, and then washed 4x1 min in PBS. Endogenous peroxidases were blocked by placing the slides in 9 mls of 3% v/v H<sub>2</sub>O<sub>2</sub>/100 mls of methanol for 5 min at R.T. The sections were then rinsed 4x1 min in PBS and then immersed in TdT buffer (See 2.6) for 2 min at R.T. The sections were then covered with 0.25 U/µl TdT and 1 nmol dUTP-biotin (both from Boehringer Mannheim) in TdT buffer and incubated in a humidified chamber at 37°C for 60 min. Negative control slides were incubated with TdT buffer alone. The reaction was stopped by transferring the slides to TB buffer (See 2.6) twice for 5 min at R.T. The sections were rinsed 4x1 min in PBS and then covered in horseradish-peroxidase-streptavidin (Dako) diluted 1:200 in house detection diluent (See 2.6). The sections were rinsed 4x1 min in PBS and then covered in chromagen (peroxidase substrate kit AEC (3-amino-9-ethyl-carbazole), Vector Lab) for 15-30 min at R.T. The reaction was followed by checking the staining occasionally under a light microscope and was stopped by rinsing the sections in ddH<sub>2</sub>O 4x1 min. The sections were then counterstained in Mayer's haematoxylin (1-2 secs), rinsed in tap H<sub>2</sub>O, immersed in STWS (turns the Mayer's haematoxylin stain blue) and rinsed finally in tap H<sub>2</sub>O. Coverslips were mounted on the sections with Supermount (Biomen Ltd.). Nuclei from apoptotic cells stain red-brown by this method. No red-brown staining could be detected of any spleen structures, from uninfected or infected mice, in negative control slides. In the positive control slides, pre-treated with DNase to nick all exposed DNA, extensive staining of all nuclei in the sections was observed.

## **2.5. Statistical analysis**

The Mann-Whitney *U*-test was used to determine the statistical significance of differences between groups. *p* < 0.05 was considered to be a significant difference.

## **2.6. Solutions**

### **2.6.1. Cell Culture**

Ammonium chloride solution: Mix 9 vols of 0.83% w/v  $\text{NH}_4\text{Cl}$  in ddH<sub>2</sub>O with 1 vol of 2.06% w/v Tris-HCl pH 7.65. pH adjusted to 7.2. Sterilized by autoclaving and stored at 4°C.

### **2.6.2. General Purpose**

PBS: 137mM NaCl, 8.1mM  $\text{Na}_2\text{HPO}_4$ , 2.7mM KCl, 1.47mM  $\text{KH}_2\text{PO}_4$  in ddH<sub>2</sub>O. pH adjusted to 7.2. Sterilized by autoclaving and stored at R.T.

### **2.6.3. ELISA Buffers**

0.06M Carbonate Buffer, pH 9.6: To 45.3ml of 1M  $\text{NaHCO}_3$  add 18.2 mls of 1M  $\text{Na}_2\text{CO}_3$  and make solution to 1 litre with ddH<sub>2</sub>O.

PBS/0.05% Tween 20: 500µl of Tween 20 was added per litre of sterile PBS.

### **2.6.4. RNA Work**

DEPC(Diethylpyrocarbonate)-treated H<sub>2</sub>O: 900µl of DEPC was added to 900mls of ddH<sub>2</sub>O and dissolved by autoclaving. Stored at R.T.

Ethidium Bromide for RNA Work: 500ng/ml stock solution diluted in DEPC-treated H<sub>2</sub>O. Stored in the dark at R.T.

Loading Buffer (5x): 10% Ficoll, 0.5% SDS, 0.06% bromophenol blue in ddH<sub>2</sub>O. Stored at R.T.

MOPS (MOPS (3-[N-Morpholino]propanesulphonic acid) buffer(10x): 0.05M Na acetate, pH 7, 0.01M EDTA, 0.2M MOPS in ddH<sub>2</sub>O. Stored at R.T. Diluted to 1x for working concentration.

### **2.6.5. DNA Work**

Denaturation Buffer: 1.5M NaCl, 0.5M NaOH in ddH<sub>2</sub>O. Stored at R.T.

Denhardt's Solution (50x): 1% ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin in ddH<sub>2</sub>O. Aliquoted and stored at -20°C.

Ethidium Bromide for DNA Work: 10mg/ml stock in ddH<sub>2</sub>O. Stored in the dark at R.T. Working concentration 100µg/ml diluted in ddH<sub>2</sub>O.

Neutralisation Buffer: 1.5M NaCl, 0.5M Tris-HCl pH7.4 in ddH<sub>2</sub>O. Stored at R.T.

SSC (20x): 3M NaCl, 0.3M Na citrate pH7.0 in ddH<sub>2</sub>O. Stored at R.T.

SSPE (20x): 3.6M NaCl, 200mM NaH<sub>2</sub>PO<sub>4</sub>, 20mM EDTA pH 7.4. Stored at R.T.

Sonicated salmon sperm DNA: DNA was sonicated and then denatured by boiling for 10 mins. Stored as a 10mg/ml stock in ddH<sub>2</sub>O at -20°C.

TAE buffer (50x): 2M Tris-HCl, 5.71% glacial acetic acid, 0.05M EDTA pH 8.2 in ddH<sub>2</sub>O. Stored at R.T. Diluted to 1x for working concentration.

TBE (10x) buffer: 0.9M Tris-HCl, 0.9M Boric acid, 25mM EDTA pH 8.3 in ddH<sub>2</sub>O. Stored at R.T. Diluted to 1x for working concentration.

### **2.6.6. FITC Staining for FACS**

DAB-2-Azide buffer: 2mls FCS and 0.1g Na azide dissolved in 100 mls of DAB (PBS with Dulbecco's A&B salts) (Gibco).

### **2.6.7. PI Staining for FACS**

Stock buffer: 10mg trisodium citrate, 0.605g, 5.22g spermine tetrahydrochloride and 20µl of Nonidet P40 (Sigma) dissolved in 100ml of dd H<sub>2</sub>O. pH adjusted to pH 7.6.

### **2.6.8. DNA Fragmentation Analysis**

DNase buffer: 10mM EDTA, 50mM Tris, pH8 in ddH<sub>2</sub>O.

Loading buffer: 10mM EDTA pH8, 1% low melting point agarose, 0.25% bromophenol blue, 40% sucrose, in ddH<sub>2</sub>O. Melt very slowly in microwave on defrost setting.

Proteinase K buffer: 10mM EDTA, 50mM Tris, pH8, 0.5% SDS in ddH<sub>2</sub>O.

### **2.6.9. TUNEL Work**

DNase buffer: 100mM DDT, 30mM Tris-HCl pH 7.2, 140mM Na cacodylate, 4mM magnesium chloride in ddH<sub>2</sub>O.

House detection diluent: 1.21g Tris, 2.92g NaCl, 0.2g magnesium chloride, adjusted to pH8.5., and 50ml Tween 20 made to 100ml with ddH<sub>2</sub>O.

TB buffer: 300mM NaCl, 30mM Na citrate in ddH<sub>2</sub>O.

TdT buffer: 30mM Tris-HCl, pH7.2, 140mM Na cacodylate, 1mM cobalt chloride in ddH<sub>2</sub>O.

## **CHAPTER 3**

## Chapter 3 Analysis of the primary immune response to s.c. infection with L3 or mf

### 3.1. Introduction

Like other helminth parasites, human filarial infection results in Th2 expansion with the consequent eosinophilia, high serum IgG4 and IgE and IL-4 production (Mahanty *et al*, 1993; Yazdanbashskh *et al*, 1993). Further investigations revealed that individuals with CP, who have generally cleared their infection, secrete IFN- $\gamma$  as well as IL-4, while actively infected individuals have the lowest levels of IgE and exhibit an antigen-specific T cell proliferative defect (Nutman *et al*, 1987a; Yazdanbashskh *et al*, 1993).

Dissecting the immunoregulatory pathways operating in man is complicated by a number of factors, including intensity of infection, exposure to different life cycle stages, host genotype, presence of other infections and pre-natal tolerance (Ottesen, 1992). Although the mouse is not fully permissive to infection with *Brugia*, single-stage infections have been used to study filarial-induced immune responses (reviewed in Lawrence, 1996).

In the first of these studies it was shown that *B. malayi* adults or mf transplanted into the peritoneal cavity of BALB/c mice survive for a number of weeks and stimulate contrasting Th cell responses (Lawrence *et al*, 1994). Implantation of adult worms polarizes the immune response in the Th2 direction. Female worms stimulate serum IgE production and very high levels of antigen-specific IL-4 from CD4<sup>+</sup> spleen cells. Adult males also stimulate antigen-specific IL-4 secretion and total IgE, but at lower levels than that induced by females. It was therefore postulated that female worms possess Th2 polarizing antigens that are likely to be derived from the uterus and may be involved in the development and release of mf. Both female and male worms induce minimal levels of antigen-specific IL-2 and IFN- $\gamma$  and significantly lower levels of

ConA-driven IFN- $\gamma$  than uninfected controls. Despite the absence of antigen-specific Th1 cytokines, spleen cells from mice implanted with adult worms proliferate strongly in response to antigen and ConA.

In contrast, BALB/c injected i.p. with *B. malayi* mf induce an early Th1 response (Pearlman *et al*, 1993; Lawrence *et al*, 1994). At day 14 p.i. CD4<sup>+</sup> spleen cells from mf infected mice secrete high levels of antigen-specific IFN- $\gamma$  (Lawrence *et al*, 1994). These mice also express elevated levels of parasite-specific IgG2a, IgG2b and IgG3. The immediate, exclusive IFN- $\gamma$  response to mf was followed by the emergence of a Th2-like response. In the study by Pearlman *et al* (1993), antigen-specific IL-5 was detected at day 14 p.i. and increased, while IFN- $\gamma$  levels declined, until day 42 when the experiment was terminated. These results are similar to those of Lawrence *et al* (1994) who detected antigen- and ConA-stimulated IL-4 and IL-5 in mf-infected mice by day 28 p.i.. Throughout the course of infection, spleen cells from mf-infected mice proliferated in response to antigen and ConA, even after the emergence of Th2 reactivity. One explanation, for the delayed Th2 response may relate to a requirement for chronic exposure/antigen restimulation (Guéry *et al*, 1996). Consistent with this hypothesis, a single immunisation with mf extract at the base of the tail results in exclusive IFN- $\gamma$  production by the draining lymph node cells, while multiple immunisation (x3) stimulates CD4<sup>+</sup> LN cells to secrete antigen-specific IL-4 and IL-5 (Pearlman *et al*, 1993).

Previous studies of Th responses elicited by the L3 in the mouse have focused on the role of Th cells in immunity against the L3. Bancroft *et al* (1993) demonstrated that successful immunization with radiation attenuated L3 of *B. pahangi* is Th2 dependent. Spleen cells from immune mice produced elevated levels of IL-4, IL-5 and IL-9 upon *in vitro* stimulation with parasite antigen or ConA. Consistent with Th2 type cytokine production, immune mice exhibited raised levels of parasite-specific IgE and a peripheral eosinophilia. *In vivo* depletion of CD4 cells, but not CD8 cells, abrogated



immunity against challenge infection and resulted in reduced secretion of parasite-specific IL-4, IL-5, and IL-9 by spleen cells and a decrease in parasite-specific serum IgG and IgE. Therefore, accelerated clearance of L3 in mice vaccinated with irradiated L3 appears to be Th2 cell dependent. In a more recent study, IL-4 KO mice were infected i.p. with *B. malayi* L3 (Lawrence *et al*, 1995). However, survival of normal L3 was not enhanced in IL-4 KO mice indicating that IL-4-dependent Th2 responses are not required for resistance against primary infection with L3 by the i.p. route. However, significant levels of antigen-specific IL-5 was still induced in IL-4 KO mice infected with L3. In the wild-type counterparts (129/Sv x C57BL/6 mice) i.p. infection with 100 L3 resulted in a Th2-type response at day 14, as evidenced by high levels of spleen cell derived IL-4, low levels of IL-5 and IL-2, but no IFN- $\gamma$  in response to antigen.

The different life cycle stages of *Brugia* therefore appear to generate contrasting Th cell responses. Stage-specific Th cell responses are not unusual in parasitic helminth infections. In murine schistosomiasis, infection with normal cercariae results in a Th1 dominated response until oviposition (Pearce *et al*, 1991). When egg-laying commences the Th1 response is downregulated and a Th2 response predominates (Sher *et al*, 1991; Pearce *et al*, 1991). Furthermore, Th2 cells are likely to be involved in granuloma formation, which, depending on the route of infection, forms around eggs trapped in the liver or the lungs (Wynn *et al*, 1994; Wynn *et al*, 1995).

The aim of these initial experiments was to characterise the response of BALB/c mice following primary infection with normal *B. pahangi* L3 by the natural (s.c.) route. Mice infected with *B. pahangi* mf were initially used as a comparative group since the early Th1 response of BALB/c mice to this stage is well characterised. Responses were analyzed at the level of proliferation, cytokine production and by measurement of antibody subclasses.

## 3.2. Results

### 3.2.1. Immune response of mice injected s.c. with L3 or mf to antigen and ConA at day 12 p.i.

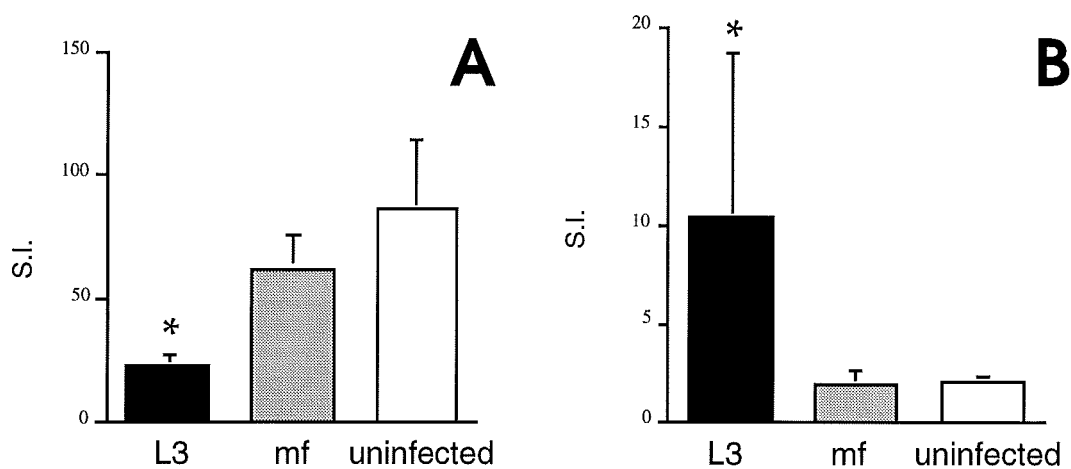
In this experiment groups of 5 BALB/c mice were injected s.c. with 50 L3,  $1 \times 10^4$  mf or HBSS (uninfected controls). At day 12 p.i. spleens were removed and serum collected for analysis.

#### Spleen cell proliferation

The proliferative responses of spleen cells from mice infected with 50 L3 or with  $1 \times 10^4$  mf or from uninfected controls to ConA and *B. pahangi* adult antigen were measured. The concentrations and culture times used (ConA  $1 \mu\text{g/ml/48h}$ ; antigen  $10 \mu\text{g/ml/72h}$ ) were shown to be optimal in preliminary experiments (data not shown).

Figure 3.1A shows that spleen cells from mice given L3 have a dramatically reduced capacity for proliferation upon *in vitro* re-stimulation with ConA compared to mice infected with mf ( $p = 0.004$ ) or to uninfected controls ( $p = 0.004$ ). In this representative experiment, the reduction in S.I. compared to uninfected controls was 73%. However, the percentage reduction in S.I. was variable between experiments, in the range 64-98%. The ConA-stimulated proliferative response of spleen cells from mf-infected mice did not differ from that of control spleen cells ( $p > 0.05$ ).

In contrast, spleen cells from L3-infected mice proliferate well in response to antigen (S.I.=10.4,  $p = 0.012$  compared to uninfected controls), whereas spleen cells from mf-infected mice were unable to respond (S.I.=1.9,  $p > 0.05$  compared to uninfected controls) (Fig. 3.1B). In two out of three additional experiments, spleen cells from mf-infected mice were able to exhibit a very low but significant proliferation in response to antigen, but always at a significantly lower level than that of L3-infected mice. Spleen cells from control animals did not proliferate in response to antigen.



**FIGURE 3.1. Proliferative responses of spleen cells from three groups of mice to ConA and *B. pahangi* adult antigen at day 12 p.i.**

Mice were injected s.c. with 50 L3,  $1 \times 10^4$  mf or HBSS (uninfected control). At day 12 p.i. proliferation of spleen cells at  $5 \times 10^6$  cells/ml to (A)  $1 \mu\text{g/ml}$  of ConA or (B)  $10 \mu\text{g/ml}$  of *B. pahangi* adult antigen was measured by uptake of  $^3\text{H}$  thymidine after 48h (ConA) or 72h (Ag) of culture. The means of triplicate wells are expressed as stimulation indices (S.I.) = cpm with ConA or antigen/cpm with medium alone. The S.I.s represent the mean  $\pm$  SD of five animals per group. \* Significant difference ( $p < 0.05$ ) compared to mice infected with mf or uninfected controls.

### **Spleen cell cytokine responses**

To investigate the significance of the contrasting proliferative responses to ConA and Ag following s.c. infection with 50 L3 or  $1 \times 10^4$  mf at day 12 p.i., IL-2 and IFN- $\gamma$  (Th1 cytokines) and IL-4, IL-5 and IL-10 (Th2 specific cytokines) were assayed. Table 3.1 shows the levels of these cytokines in 48hr ConA- and antigen-stimulated supernatants produced by spleen cells from the three groups of mice. Cells incubated in medium alone do not produce any of the cytokines measured. Consistent with the reduced mitogen stimulated proliferation, mice infected with L3 secrete significantly less IL-2 in response to ConA than uninfected controls ( $p = 0.004$ ). Spleen cells from these animals also produce less IFN- $\gamma$  when stimulated with ConA ( $p = 0.004$ ), in contrast to spleen cells from mice infected with mf, that secrete levels of IL-2 ( $p > 0.05$ ) and IFN- $\gamma$  ( $p > 0.05$ ) equivalent to those of uninfected controls. However, spleen cells from mice given L3 are not completely unresponsive to ConA since they produce elevated levels of IL-4, IL-5 and IL-10 in comparison to mice infected with mf or uninfected controls ( $p = 0.004$  in all cases). In terms of antigen-driven cytokine responses, mice infected with L3 did not secrete any IFN- $\gamma$  and insignificant levels of IL-2 ( $p > 0.05$  compared to uninfected controls), but were the only group to produce antigen-specific IL-4, IL-5, and IL-10. In contrast, spleen cells from mice infected with mf, while unable to produce any antigen-specific IL-4, IL-5 or IL-10 ( $p > 0.05$  for all the cytokines) and only minimal IL-2 ( $1.8 \pm 0.8$  U/ml; assay sensitivity = 0.781 U/ml), produced IFN- $\gamma$  exclusively.

### **Parasite-specific serum antibody isotype responses**

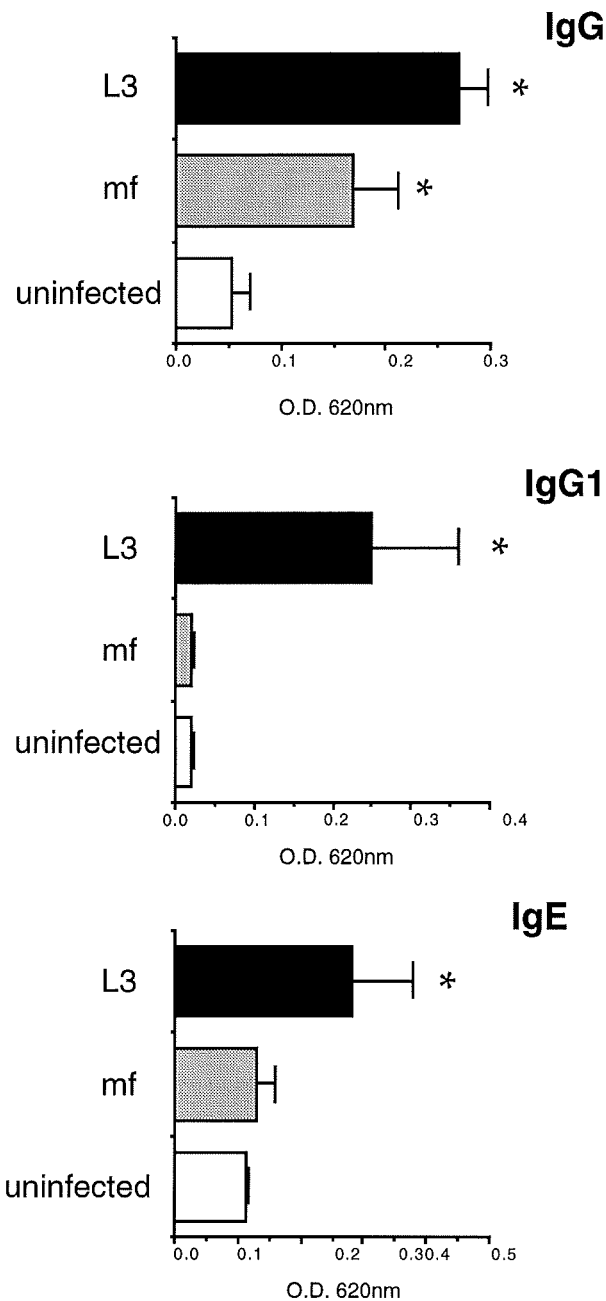
Parasite-specific antibody isotype responses in serum following infection with 50 L3 or  $1 \times 10^4$  mf were measured as *in vivo* correlates of Th cell activity (Fig. 3.2). Consistent with the Th2 activation revealed by cytokine analysis, L3 also stimulate significant levels of parasite-specific IgG1 and IgE ( $p = 0.004$  for both isotypes compared to uninfected controls), but not IgG2a ( $p > 0.05$ , data not shown). Mf-infected mice produced significant level of parasite-specific IgG ( $p = 0.004$ ). Despite the production

**TABLE 3.1. Cytokine responses of spleen cells from three groups of mice<sup>a</sup> to ConA and *B. pahangi* adult antigen at day 12 p.i.**

stimulus	cytokine levels (U/ml) <sup>b</sup>			
	L 3	Mf	uninfected	
Ag	1.1 ± 0.8 (NS)	1.8 ± 0.8 *	0	<b>IL-2</b>
ConA	28.7 ± 12.1 *	71.1 ± 12.3	79.1 ± 8.7	
Ag	0	27.8 ± 8.7 *	0	<b>IFN-γ</b>
ConA	184.5 ± 28.4 *	338.1 ± 63.3	314.8 ± 33.2	
Ag	0.6 ± 0.2 *	0	0	<b>IL-4</b>
ConA	28.7 ± 12.4 *	5.0 ± 1.5 (NS)	11.0 ± 7.0	
Ag	13.5 ± 2.7 *	0	0	<b>IL-5</b>
ConA	25.6 ± 8.7 *	2.6 ± 1.4 (NS)	2.7 ± 0.8	
Ag	0.8 ± 0.6 *	0	0	<b>IL-10</b>
ConA	1.9 ± 1.2 *	0	0	

<sup>a</sup> Mice were injected s.c. with 50 L3, 1x10<sup>4</sup> mf or HBSS (uninfected).

<sup>b</sup> At day 12 p.i. cytokine levels in 48h supernatants from spleen cells at 1x10<sup>7</sup> cells/ml in response to ConA (5µg/ml) or *B. pahangi* adult antigen (10µg/ml) were measured by 2-site ELISA with reference to standards of known concentration (U/ml). A value of 0 indicates a reading below the sensitivity of the assay. The values are means ± SD of five animals per group. \* Significant difference ( $p < 0.05$ ) between infected mice and uninfected controls. NS;  $p > 0.05$ .



**FIGURE 3.2. Antigen-specific antibody isotype levels in the serum of three groups of mice at day 12 p.i.**

Animals were injected s.c. with 50 L3,  $1 \times 10^4$  mf or HBSS (uninfected control). Ag-specific antibody levels for IgG, IgG1 and IgE, in serum collected at day 12 p.i., were measured by ELISA using *B. pahangi* adult extract as the target antigen. The values represent the means  $\pm$  S.D. of five animals per group. \* Significant difference ( $p < 0.05$ ) compared to uninfected controls. IgG levels were also significantly different ( $p < 0.05$ ) between L3-infected and mf infected mice.

of antigen-specific IFN- $\gamma$  by spleen cells from mf-infected mice no serum IgG2a could be detected ( $p > 0.05$ , data not shown). However, mice infected with a higher dose of mf ( $1 \times 10^5$ ) produce elevated levels of parasite-specific IgG2a (see Expt. 3.2.6). The highest level of parasite-specific IgG was consistently detected in mice infected with L3, compared to mf ( $p = 0.004$ ), perhaps a reflection of the superior B cell help provided by Th2 cells in these mice.

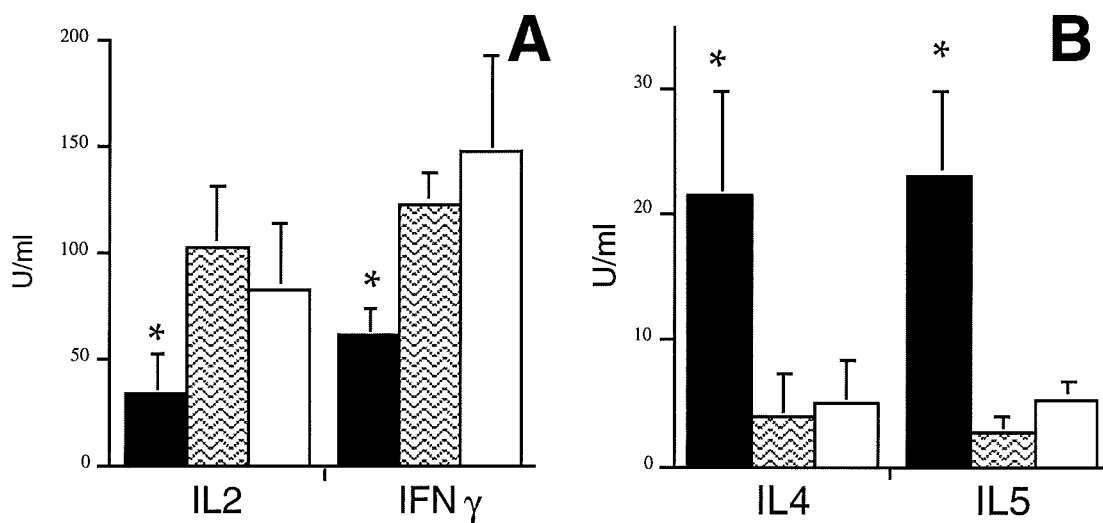
### **3.2.2. Comparison of the immune responses of mice injected s.c. with live versus dead L3 at day 12 p.i.**

The effect on mitogen driven Th1 responses at day 12 p.i. appears to be L3-specific since mf do not down-regulate these responses. To further investigate the nature of the L3-specific antigens that may be involved, cytokine responses stimulated by 50 live L3 versus 50 dead (heat-killed) L3 were compared at day 12 p.i. (Fig. 3.3). These experiments show that unlike live L3, dead L3 have no effect on ConA stimulated IL-2 or IFN- $\gamma$  production ( $p > 0.05$  for both cytokines) (Fig. 3.3A), nor do they stimulate secretion of the Th2 cytokines, IL-4 or IL-5 ( $p > 0.05$  for both cytokines) (Fig. 3.3B).

### **3.2.3. Effect of route of infection on Th responses elicited by infection with L3 or mf at day 12 p.i.**

Since the nature of the Th cell response can be influenced by the route of antigen entry (Bretscher *et al*, 1992), the responses of mice, infected with 50 L3 or  $1 \times 10^4$  mf by the s.c. and i.p. route, were compared at day 12 p.i.. Control mice were given a s.c. injection of HBSS.

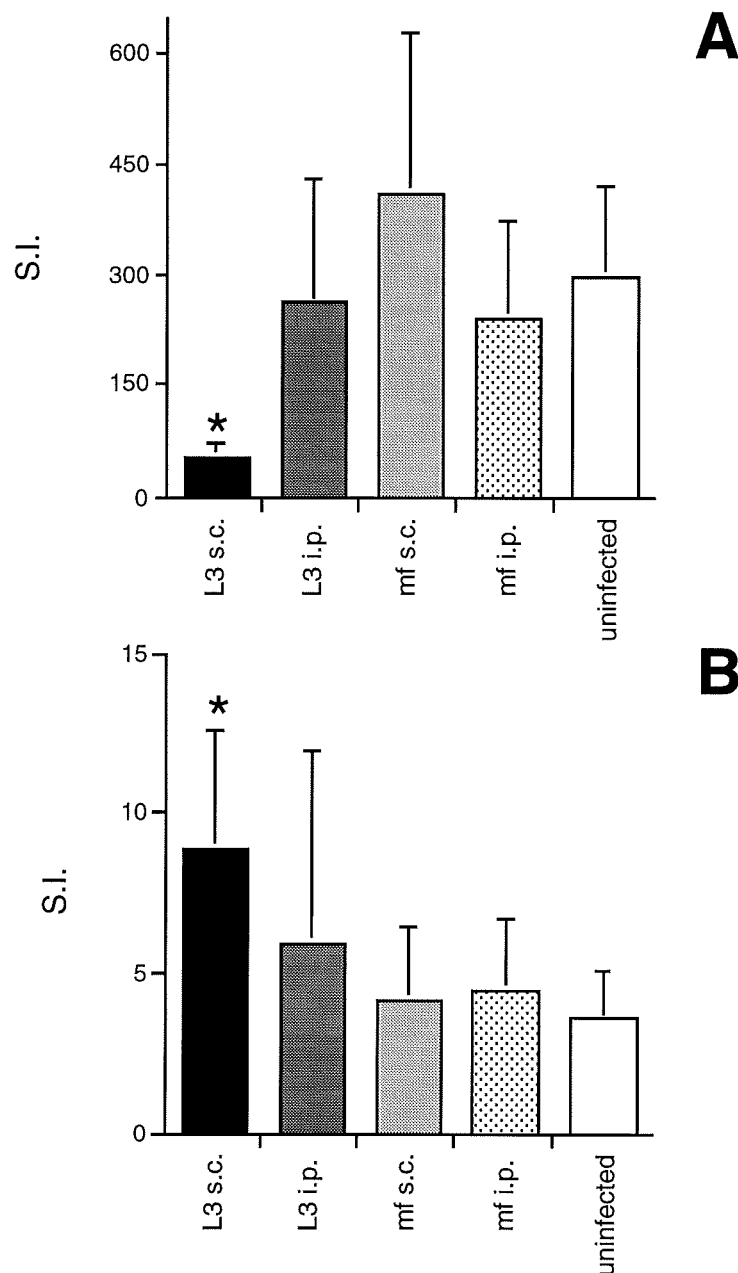
This experiment demonstrates that unlike s.c. infection with L3, i.p. infection fails to down-regulate the ConA-driven proliferative, IL-2 and IFN- $\gamma$  responses of spleen cells or to stimulate their secretion of IL-4, IL-5 or IL-10 in response to antigen (Fig. 3.4A and Table 3.2). However, L3 injected i.p. were able to provoke a weak Th2-type response as signified by the production of ConA-driven IL-4 and IL-10 ( $p = 0.004$  for



**FIGURE 3.3. ConA-stimulated spleen cell cytokine production after infection with live versus dead L3.**

Mice were injected s.c. with 50 live L3 ■, 50 dead (heat-killed) L3 ▨ or HBSS (uninfected control) □. At day 12 p.i. levels of IL-2 and IFN- $\gamma$  (A), IL-4 and IL-5 (B) in 48h supernatants from spleen cells at  $1 \times 10^7$  cells/ml in response to ConA ( $5 \mu\text{g/ml}$ ) were measured by ELISA. The values represent the means  $\pm$  S.D. of five animals per group. \* Significant difference ( $p < 0.05$ ) between infected mice and uninfected controls.





**FIGURE 3.4. Proliferative responses of spleen cells from five groups of mice following s.c. or i.p. injection with L3 and mf to ConA and *B.pahangi* adult antigen.**

Mice were injected s.c. or i.p. with 50 L3 and  $1 \times 10^4$  mf. Uninfected controls were injected s.c. with HBSS. At day 12 p.i. proliferation of spleen cells at  $5 \times 10^6$  cells/ml to (A)  $1 \mu\text{g/ml}$  of ConA or (B)  $10 \mu\text{g/ml}$  of *B. pahangi* adult antigen was measured by uptake of  $^3\text{H}$  thymidine after 48h (ConA) or 72h (antigen) of culture. The means of triplicate wells are expressed as stimulation indices (S.I.) = cpm with ConA or antigen/cpm with medium alone. The S.I.s represent the mean  $\pm$  SD of 5 animals per group. \* Significant difference ( $p < 0.05$ ) compared to uninfected controls.

**TABLE 3.2. Cytokine responses of spleen cells from five groups of mice<sup>a</sup> to ConA and *B. pahangi* adult antigen at day 12 p.i.**

stimulus	cytokine levels (U/ml) <sup>b</sup>					
	L3 s.c.	L3 i.p.	Mf s.c.	Mf i.p.	uninfected	
Ag	1.99±0.9*	2.3±1.5*	1.7±0.6*	1.8±0.5*	0	<b>IL-2</b>
ConA	37.4±15.3*	95.6±37.1	100.5±28.2	124.2±53.0	89.4±20.5	
Ag	0	0	20.8±7.7*	23.3±12.1*	0	<b>IFN-γ</b>
ConA	62.6±31.7*	200.6±140.1	164.6±43.5*	162.4±73.3*	93.1±13.4	
Ag	0.7±0.3*	0	0	0	0	<b>IL-4</b>
ConA	20.8±6.6*	13.5±3.0*	7.7±0.9	9.4±3.7	6.8±0.4	
Ag	9.6±2.1*	0	0	0	0	<b>IL-5</b>
ConA	13.5±3.9*	0	0	0	0	
Ag	0.4±0.2*	0	0	0	0	<b>IL-10</b>
ConA	0.6±0.3*	0.3±0.07*	0	0	0.2±0.02	

<sup>a</sup> Mice were injected s.c. or i.p. with 50 L3, 1x10<sup>4</sup> mf or s.c. with HBSS (uninfected).

<sup>b</sup> At day 12 p.i. cytokine levels in 48h supernatants from spleen cells at 1x10<sup>7</sup> cells/ml in response to ConA (5µg/ml) or *B. pahangi* adult antigen (10µg/ml) were measured by 2-site ELISA with reference to standards of known concentration (U/ml). A value of 0 indicates a reading below the sensitivity of the assay. The values are means ± SD of five animals per group. \* Significant difference ( $p < 0.05$ ) between infected mice and uninfected controls. NS;  $p > 0.05$ .

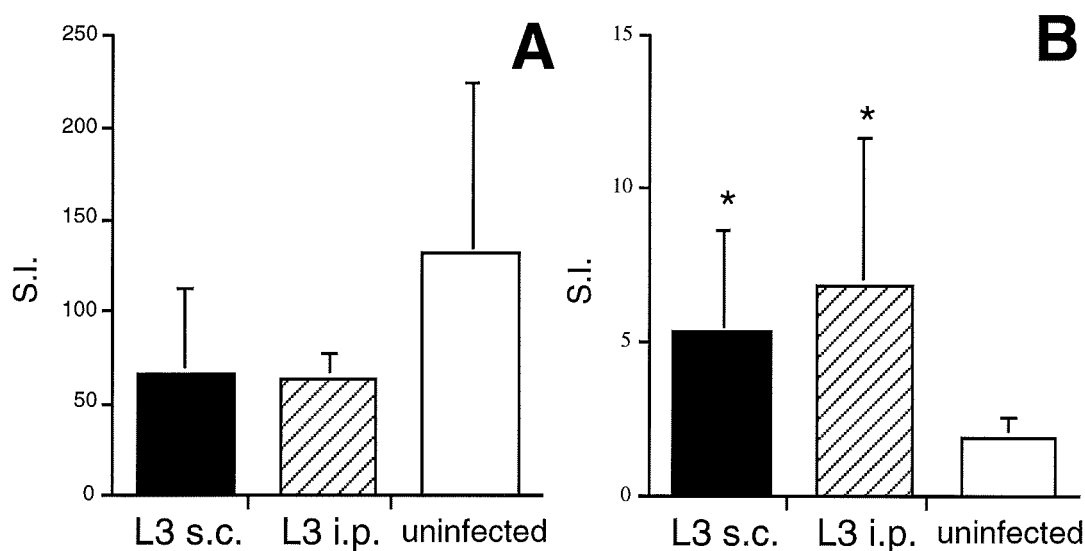
both cytokines, compared to uninfected controls), but at significantly lower levels than mice infected with L3 by the s.c. route ( $p = 0.016$  for IL-4,  $p = 0.004$  for IL-10) (Table 2.2). In the experiment shown in Fig. 3.4B spleen cells from mice infected i.p. with L3 were unable to proliferate in response to antigen (S.I.=5.9,  $p > 0.05$  compared to uninfected controls), unlike spleen cells from s.c. infected animals (S.I.=8.9,  $p = 0.004$  compared to uninfected controls). However, two out of five of the mice infected i.p. with L3 exhibited significant antigen-stimulated proliferation compared to uninfected controls. In addition, in five out of six repeat experiments, spleen cells from mice infected i.p. with L3 were able to exhibit a low but significant level of antigen-specific proliferation but at a reduced level compared to that of s.c infected mice.

The route of infection did not appear to influence the dominant Th1-type response to mf. Mice infected with mf by either route exhibited normal spleen cell proliferation and IL-2 production in response to ConA (Fig. 3.4A and Table 3.2). In addition, antigen- and ConA-stimulated IFN- $\gamma$  production was elevated only in mice infected with mf by either route (Table 3.2). Furthermore, antigen-specific T cell proliferation was absent following infection with  $1 \times 10^4$  mf by either route ( $p > 0.05$  in both cases, compared to uninfected controls) (Fig. 3.4B).

#### **3.2.4. Cytokine and proliferative responses of splenocytes from mice injected s.c. with L3 to antigen and ConA at day 4 p.i.**

In this experiment ConA-stimulated proliferation and cytokine responses of spleen cells at day 4 p.i. with L3 was measured to determine whether the Th imbalance was apparent early in infection. Mice were injected s.c. or i.p. with L3. Control mice were given a s.c. injection of HBSS.

The results presented in Fig. 3.5 and Table 3.3 show that the level of ConA-stimulated proliferation and IL-2 and IFN- $\gamma$  production in L3-infected mice are not significantly different from those of uninfected control mice ( $p > 0.05$  in all cases). Antigen-primed



**FIGURE 3.5. Proliferative responses of spleen cells from L3-infected mice to ConA and *B. pahangi* adult antigen at day 4 p.i..**

Mice were injected s.c. ■ or i.p. ▨ with 50 L3 or s.c. with HBSS □ (uninfected control). At day 4 p.i. proliferation of spleen cells at  $5 \times 10^6$  cells/ml to (A)  $1 \mu\text{g/ml}$  of ConA or (B)  $10 \mu\text{g/ml}$  of *B. pahangi* adult antigen was measured by uptake of  $^3\text{H}$  thymidine after 48h (ConA) or 72h (antigen) of culture. The means of triplicate wells are expressed as stimulation indices (S.I.) = cpm with ConA or antigen/cpm with medium alone. The S.I.s represent the mean  $\pm$  SD of 5 animals per group. \* Significant difference ( $p < 0.05$ ) compared to uninfected controls.

**TABLE 3.3. Cytokine responses of spleen cells from three groups of mice<sup>a</sup> to ConA at day 4 p.i.**

cytokine levels (U/ml) <sup>b</sup>			
L3 s.c.	L3 i.p.	uninfected	
80.4±19.4	105.5±53.5	57.8±16.7	<b>IL-2</b>
123.8±5.8	121.9±16.7	111.7±9.9	<b>IFN-γ</b>
0.28±0.07	0.25±0.04	0.28±0.08	<b>IL-4</b>
3.8±1.6	3.8±1.1	4.3±4.3	<b>IL-5</b>
0.9±0.4	1.2±0.4	1.0±0.2	<b>IL-10</b>

<sup>a</sup> Mice were injected s.c. or i.p. with 50 L3 or s.c. with HBSS (uninfected).

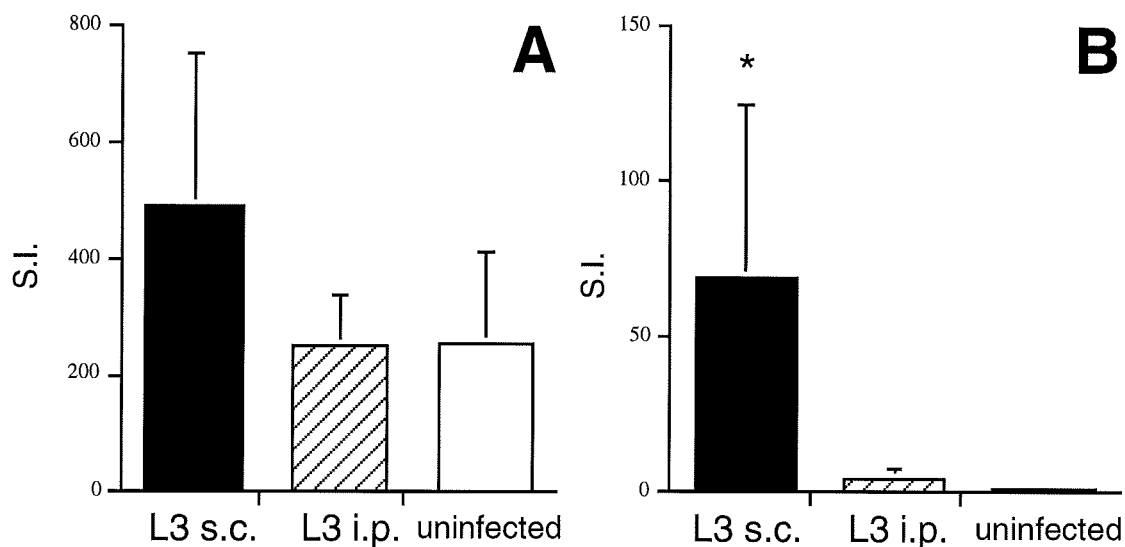
<sup>b</sup> At day 4 p.i. cytokine levels in 48h supernatants from spleen cells at  $1 \times 10^7$  cells/ml in response to ConA (5μg/ml) were measured by 2-site ELISA with reference to standards of known concentration (U/ml). The values are means ± SD of five animals per group.

cells were present in the spleen cell populations from both groups of L3-infected mice, at day 4 p.i., since a significant level of antigen-specific proliferation could be measured (L3 s.c. S.I.= 5.3 and L3 i.p. S.I.=6.8,  $p = 0.004$  compared to uninfected controls in both cases). However, no Th2 cytokines (supernatants were assayed for the presence of IL-4, IL-5 and IL-10) could be detected following antigen or ConA stimulation of spleen cells from L3-infected mice.

### **3.2.5. Cytokine and proliferative response of mice injected with L3 at day 19 p.i.**

To assess the permanence of the Th1 down-regulated responses in mice infected s.c. with L3, the immune response was analyzed at day 19 p.i.. Mice were injected with 50 L3 either s.c. or i.p. Control mice were given a s.c. injection of HBSS.

By day 19 p.i. the levels of Con A-stimulated proliferation (Fig. 3.6A) and IL-2 and IFN- $\gamma$  produced (Table 4) by spleen cells from mice infected s.c. with L3 are restored to the level of uninfected controls ( $p > 0.05$  in all cases). This restoration of polyclonal Th1 responsiveness occurs in the presence of the continued production of elevated levels of antigen- and ConA-stimulated IL-4 and IL-5 compared with uninfected controls (Table 3.4). Spleen cells from mice infected s.c. with L3 still proliferate vigorously in response to antigen at day 19 (S.I.=69.0,  $p = 0.004$  compared to uninfected controls) whereas cells from i.p. infected mice do not (S.I.=4.6,  $p > 0.05$  compared to uninfected controls) (Fig. 3.6B). However, in contrast to the situation at day 12 p.i., spleen cells from mice infected s.c. with L3 produce significant levels of antigen-specific IFN- $\gamma$  (Table 3.4). In addition, spleen cells from mice infected i.p. with L3 secrete equivalent levels of ConA-stimulated IL-4 to that of s.c. infected mice (Table 3.4). However, no increase in IL-5 or IL-10 secretion by spleen cells from mice infected i.p. could be detected and ConA-driven IL-2 and IFN- $\gamma$  production was not significantly different from uninfected controls. In addition, levels of parasite-specific



**FIGURE 3.6. Proliferative responses of spleen cells from L3-infected mice to ConA and *B. pahangi* adult antigen at day 19 p.i..**

Mice were injected s.c. ■ or i.p. ▨ with 50 L3 or s.c. with HBSS □ (uninfected control). At day 19 p.i. proliferation of spleen cells at  $5 \times 10^6$  cells/ml to (A)  $1 \mu\text{g/ml}$  of ConA or (B)  $10 \mu\text{g/ml}$  of *B. pahangi* adult antigen was measured by uptake of  $^3\text{H}$  thymidine after 48h (ConA) or 72h (antigen) of culture. The means of triplicate wells are expressed as stimulation indices (S.I.) = cpm with ConA or antigen/cpm with medium alone. The S.I.s represent the mean  $\pm$  SD of 5 animals per group. \* Significant difference ( $p < 0.05$ ) compared to uninfected controls.

**TABLE 3.4. Cytokine responses of spleen cells from three groups of mice<sup>a</sup> to ConA and *B. pahangi* adult antigen at day 19 p.i.**

stimulus	cytokine levels (U/ml) <sup>b</sup>			
	L3 s.c.	L3 i.p.	uninfected	
Ag	1.9 ± 1.1	2.0 ± 0.4 *	1.0 ± 0.5	<b>IL-2</b>
ConA	49.9 ± 12.6	65.5 ± 14.6	77.2 ± 24.3	
Ag	14.4 ± 8.0*	0	0	<b>IFN-γ</b>
ConA	126.7 ± 12.7	122.4 ± 14.8	124.8 ± 16.4	
Ag	0.45 ± 0.33*	0	0	<b>IL-4</b>
ConA	11.6 ± 3.1*	8.7 ± 12.2	4.0 ± 1.8	
Ag	13.6 ± 7.0*	1.4 ± 1.1	0	<b>IL-5</b>
ConA	17.2 ± 9.4 *	1.5 ± 0.6	0.9 ± 0.2	
Ag	1.6 ± 0.5*	0	0	<b>IL-10</b>
ConA	0.26 ± 0.03*	0.22 ± 0.07	0.14 ± 0.06	

<sup>a</sup> Mice were injected s.c. with 50 L3, 1x10<sup>4</sup> mf or HBSS (uninfected).

<sup>b</sup> At day 19 p.i. cytokine levels in 48h supernatants from spleen cells at 1x10<sup>7</sup> cells/ml in response to ConA (5µg/ml) or *B. pahangi* adult Ag (10µg/ml) were measured by 2-site ELISA with reference to standards of known concentration (U/ml). A value of 0 indicates a reading below the sensitivity of the assay. The values are means ± SD of 5 animals per group. \* Significant difference ( $p < 0.05$ ) between infected mice and uninfected controls.



IgG and IgE in s.c. infected mice remain significantly higher than in i.p. infected animals (data not shown).

### **3.2.6. Analysis of primary response to s.c. infection with increased doses of mf at day 12 p.i.**

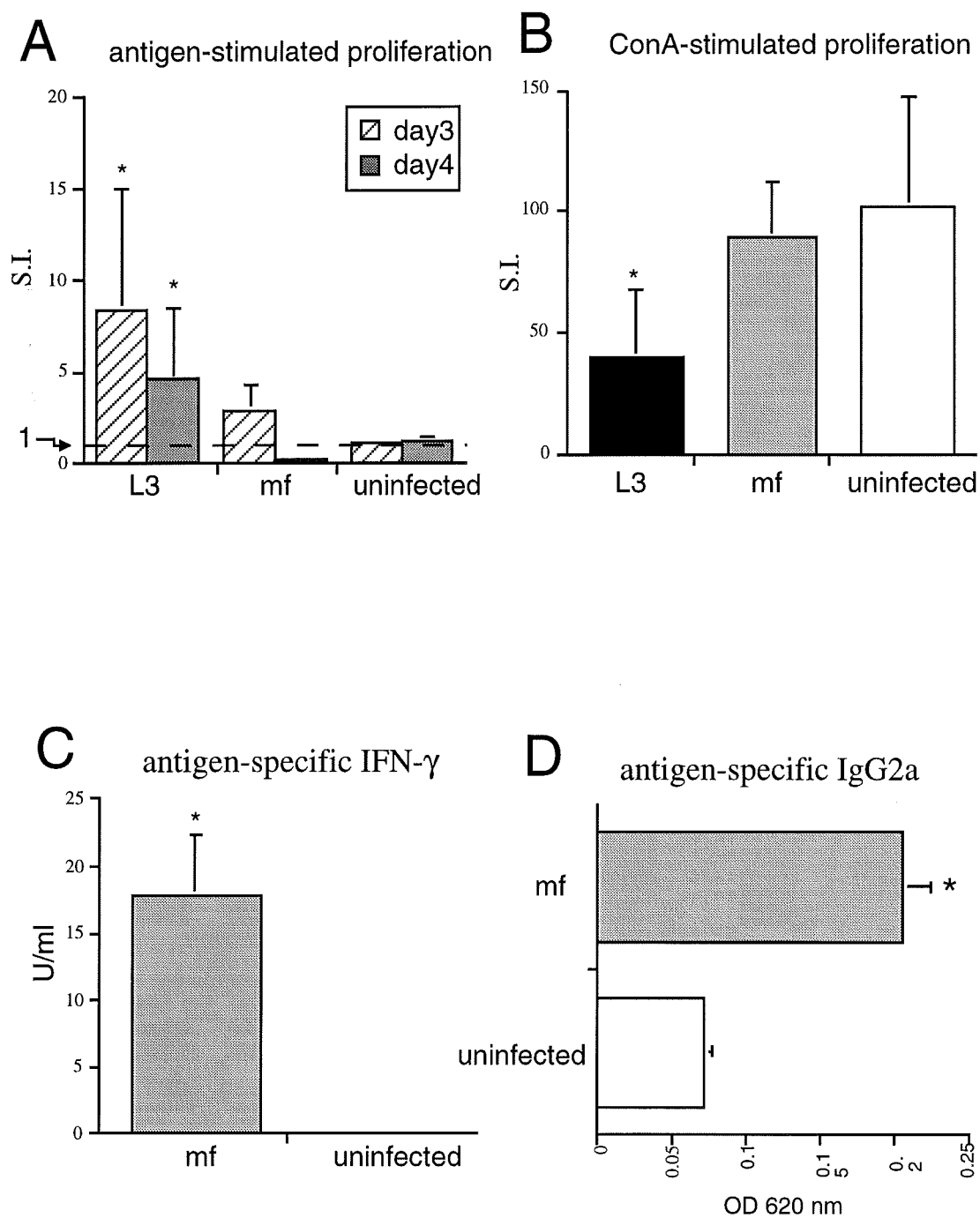
In mice infected s.c. with  $10^4$  mf, antigen-proliferative spleen cell responses were mostly absent and parasite-specific IgG2a was undetectable at day 12 p.i. (Expts. 3.2.1 and 3.2.3). However, i.p. infection with  $2 \times 10^5$  *B. malayi* mf results in antigen-driven spleen cell proliferation at day 14 p.i. and parasite-specific serum IgG2a production at day 21 p.i. (Lawrence *et al*, 1994). Since the direct comparison of spleen cell responses elicited by L3 infected s.c. versus i.p. (Expts. 3.2.3 and 3.2.5), appear to demonstrate that s.c. infection is the more potent route of immunisation, it was decided to infect mice s.c. with  $1 \times 10^5$  mf. In this experiment mice were injected s.c. with 50 L3,  $1 \times 10^5$  mf or HBSS and proliferation, cytokine production and antibody responses measured at day 12 p.i.

Spleen cells from mice infected s.c. with  $1 \times 10^5$  mf produce high levels of antigen-driven IFN- $\gamma$ , display normal ConA-driven proliferation (Fig 3.7C & B), IL-2 and IFN- $\gamma$  production (data not shown). Spleen cells from mice infected s.c. with  $1 \times 10^5$  mf do not secrete antigen- or ConA-stimulated Th2 cytokines (data not shown). In addition, s.c. infection with  $1 \times 10^5$  mf stimulates high levels of parasite-specific serum IgG2a ( $p = 0.004$ ) (Fig. 3.7D). However, spleen cells from mice given  $1 \times 10^5$  mf are unable to respond to antigen after 3 days in culture (S.I. mf= $2.9 \pm 1.4$  v S.I. control= $1.1 \pm 0.1$ ,  $p > 0.05$ ), in contrast to spleen cells from L3 infected mice that respond strongly (S.I. L3= $8.4 \pm 6.6$ ,  $p = 0.004$ ) (Fig. 3.7A). In repeated experiments, the S.I. of spleen cells from mice infected with  $10^5$  mf to antigen was either very low (four out of seven) or not significantly different (three out of seven) from uninfected controls and was always significantly lower than that of L3 infected mice. When antigen-stimulated proliferation was measured after 4 days in culture the S.I. of  $10^5$  mf-infected mice was  $<1$  ( $0.3 \pm 0.1$ )

**FIGURE 3.7. Immune responses of mice infected with  $1 \times 10^5$  mf at day 12 p.i.**

Mice were injected s.c. with 50 L3,  $1 \times 10^5$  mf or HBSS (uninfected control). At day 12 p.i. proliferation of spleen cells at  $5 \times 10^6$  cells/ml to (A)  $1 \mu\text{g/ml}$  of ConA at 48h or (B)  $10 \mu\text{g/ml}$  of *B. pahangi* adult antigen at 72h and 96h was measured by uptake of  $^3\text{H}$  thymidine. The means of triplicate wells are expressed as stimulation indices (S.I.) = cpm with ConA or antigen/cpm with medium alone. The S.I.s represent the mean  $\pm$  SD of 5 animals per group. (C) Level of IFN- $\gamma$  in 48h supernatants from spleen cells at  $1 \times 10^7$  cells/ml in response to antigen ( $10 \mu\text{g/ml}$ ), measured by ELISA. A value of 0 indicates a reading below the sensitivity of the assay,  $\geq 0.195$  U/ml. (D) Level of parasite-specific serum IgG2a, measured by ELISA using *B.pahangi* adult extract as the target antigen. All values represent the means  $\pm$  S.D. of 5 animals per group.\* Significant difference ( $p < 0.05$ ) compared to uninfected controls.

**Figure 3.7.**



and significantly less than the S.I. of cells from uninfected controls ( $p = 0.008$ ) (Fig. 3.7A). The S.I. of cells from uninfected mice at day 4 was  $>1$  (S.I.=1.2). After 4 days in culture the S.I. of spleen cells from L3-infected mice in response to antigen was significantly lower than after 3 days (S.I.= $4.6 \pm 3.9$ ,  $p = 0.028$ ). However, spleen cells from L3-infected were still responding significantly to antigen after 4 days ( $p = 0.048$ ).

### 3.3. Discussion

The results presented in this chapter indicate that both *B. pahangi* L3 and mf given s.c. have very powerful immunomodulatory effects on primary Th cell responses. Infection of BALB/c mice with L3 induces a Th2 dominated response at day 12 p.i. such that Th1 responses are severely impaired. In contrast, infection with mf results in an exclusive Th1-type response at day 12 p.i. However, spleen cells from mf-infected mice are unable to proliferate in response to antigen. Therefore, analysis of the primary response to single stages has replicated some aspects of the human infection. Actively infected humans, who by definition have been exposed to all life cycle stages, generally exhibit a Th2 biased response and an antigen-specific proliferative defect.

At day 12 p.i. splenocytes from mice infected with L3 secreted elevated levels of IL-4, IL-5, and IL-10 in response to ConA but were unable to proliferate normally or to produce equivalent levels of IL-2 and IFN- $\gamma$  in comparison to uninfected mice. The selective expansion of Th2 cells is reflected *in vivo* by the expression of parasite-specific antibody of the IgE and IgG1 isotypes but not IgG2a. While other cell types are able to produce Th2-type cytokines during the induction phase of an immune response, antigen-specific IL-4, IL-5, and IL-10 detected following s.c. infection with L3 is likely to derive from CD4<sup>+</sup> cells. Further experiments examining the response elicited by L3 following injection into the footpad demonstrated that antigen-specific IL-4, IL-5, and IL-10 are produced exclusively by CD4<sup>+</sup> LN cells at day 10 p.i. (see Expt. 5.2.5). In addition, CD4<sup>+</sup> cells were the major source of parasite-driven IL-4, IL-5 and IL-9 at later time points in mice immunised with radiation attenuated L3 (Bancroft *et al*, 1994a).

The impaired polyclonal proliferative response observed in L3-infected mice suggested that antigen-driven proliferation might be similarly down-regulated; however, this was not the case. Spleen cells from mice infected with L3 retained the capacity to proliferate in response to specific antigen but only Th2 cytokines were secreted and neither IL-2

nor IFN- $\gamma$  was detectable. Therefore, although the demonstration of antigen-stimulated proliferation would, at first sight, indicate that filarial-specific responses were intact, closer examination reveals that only one arm of the immune response (i.e. Th2) is activated and that antigen-specific Th1 cells are absent or have been rendered unresponsive.

The down-regulation of polyclonal Th1 responses is restricted to infection with L3, as cells from mf infected mice proliferated normally and produced IL-2 and IFN- $\gamma$  in response to ConA. Antigen levels are known to influence the type of Th cell response elicited (Constant *et al*, 1995; Hosken *et al*, 1995; Guéry *et al*, 1996) and in one study with the nematode parasite *T. muris*, higher doses of antigen favoured the development of a Th2 response (Bancroft *et al*, 1994). However, this explanation is not sufficient to account for the different immune responses induced by mf and L3 since the dose of mf given, which induced a Th1-like response, constituted a greater parasite mass than the L3. In addition, L3 will undergo development to the L4 stage in the mouse and thus provide a continuing source of antigen, while mf cannot develop further in the mammalian host. Heat-killed L3 were also unable to impair Th1 responses or to stimulate a Th2 response, implying that the important L3 antigens are heat labile or are released in sufficient quantity by the L3 or L4. Alternatively, antigens released from living parasites may be differentially processed or presented, perhaps due to parasite migration from the site of inoculation.

Route of inoculation can influence the induction of T cell unresponsiveness (Aichele *et al*, 1995) and the nature of the Th cell response that develops (Bretscher *et al*, 1992), which is thought to reflect differential targetting of professional APCs. Therefore, as an alternative route of antigen presentation, mice were infected i.p. with L3 and mf. In these experiments, L3 given i.p. were unable to down-regulate polyclonal Th1 responses in the spleen. In addition, spleen cells from these mice were able to mount only a weak Th2 response to the parasite. Polyclonal Th1 responses were intact in mice

infected with mf by either route. The results suggest that the magnitude of the Th2 response elicited by L3 is important in down-regulating polyclonal Th1 responses in the spleen. Consistent with this observation, mitogen-driven Th1 responses are normal at day 4 p.i. with L3, prior to the induction of a measurable Th2 response. In addition, despite the late appearance of a weak Th2 response in mice infected with L3 by the i.p. route at day 19 p.i., there was no coincident reduction in mitogen-driven Th1 cytokine production. Therefore, it appears that APCs do have a role in down-regulating Th1 responses following s.c infection with L3.

It has been shown that *B. malayi* adult worms implanted into the peritoneal cavity inhibit antigen- and ConA-induced proliferation of peritoneal T cells (Allen *et al*, 1996) but not spleen cells (Lawrence *et al*, 1994). Therefore, it may be necessary to examine the responses of T cells that are present at the site of infection to gain a more accurate impression of T cell responsiveness in mice infected i.p. with L3. However, since the effect of i.p. infection with L3 on peritoneal T cell proliferation was not assessed in the study by Allen *et al* (1996), it is not possible to directly compare the inhibition of T cell responses by adults versus L3, the two stages that elicit a similar Th2 biased cytokine profile. Spleen cells from mice implanted with either female or male worms do secrete significantly lower levels of ConA-driven IFN- $\gamma$  at day 14 p.i. than uninfected controls (Lawrence *et al*, 1994), which would imply some level of polyclonal Th1 down-regulation in the spleen following i.p. filarial infection. The inability to detect a proliferative defect in the spleens of these mice in response to mitogen may be due to the concentration of ConA and culture time that was used in that study (5 $\mu$ g/ml ConA; 72h). In the experiments in this chapter, the concentration of ConA used for the proliferation assay (1 $\mu$ g/ml) is five times lower than for cytokine production and the assay time is 48h. If the concentration is increased or culture time altered, then the suppressed response of spleen cells from mice infected s.c. with L3 is significantly reduced.

Spleen cells from jirds chronically infected with *B. pahangi* exhibit markedly suppressed mitogen-induced proliferation and IL-2 production in comparison to uninfected controls (Portaro *et al*, 1976; Lammie and Katz, 1983; Leiva and Lammie, 1989). The responsiveness of spleen cells from infected animals to the T cell mitogens PHA and Con A was found to be reduced by up to 90% when compared to the reactivity of splenocytes from uninfected animals (Lammie and Katz, 1983). This level of reduction is within the limits observed for splenocytes from L3-infected mice (60-90%). However, mitogen non-responsiveness is related to the onset of microfilaraemia in the jird.

By day 19 following s.c infection with L3, Th1 mitogen-driven responses were restored to the level of uninfected mice while Th2 responses remain elevated. The fate of L3 given by the scruff-of-the-neck is not known. However, when L3 are given i.p. approximately 40% of developing L4/adults are recovered from the peritoneal cavity of BALB/c mice up to day 19 p.i. (Howells *et al*, 1983). Therefore, the late restoration of defective responses following primary infection may reflect the requirement for live parasites for Th1 down-regulation, as demonstrated in experiment 2. In addition, antigen-specific IFN- $\gamma$  was detected at day 19 p.i. (but not at day 12 p.i.) suggesting that the Th2 response was in decline and providing further evidence that a strong Th2 activity is required to suppress mitogen-driven Th1 responses. It would be interesting to discover if long-term polyclonal Th1 unresponsiveness could be achieved following repeated infection with L3.

The conclusion from the experiments with the L3, is that the impairment of mitogen-driven Th1 responses in the spleen following s.c. infection appears to be dependent upon the development of a Th2 polarized response. Since this may also reflect the nature of APC targetted by this route, the influence of both Th2 cytokine production and the APC population on generating Th1 unresponsiveness to mitogen following s.c. infection with L3 is further investigated in Chapter 4.



While displaying normal mitogen-stimulated Th1 responsiveness, spleen cells from mice infected s.c. or i.p. with  $1 \times 10^4$  mf are unable to proliferate in response to antigen or to produce detectable IgG2a. However, spleen cells from these mice are clearly activated by infection as demonstrated by high levels of antigen-specific IFN- $\gamma$ . In the study of Lawrence *et al* (1994), i.p. infection of BALB/c mice with  $2 \times 10^5$  *B. malayi* mf resulted in high levels of parasite-specific serum IgG2a and spleen cells from these mice proliferated strongly and secreted IFN- $\gamma$  in response to antigen at day 14 p.i. To ensure that the absence of antigen-specific proliferation and IgG2a was not simply due to insufficient antigen priming with a dose of  $1 \times 10^4$  mf, mice were also infected with  $10^5$  mf. Spleen cells from these mice also produced high levels of antigen-specific IFN- $\gamma$  and displayed normal ConA-stimulated proliferative and IFN- $\gamma$  responses. In addition, mice infected with  $10^5$  mf produced high levels of parasite-specific IgG2a. However, despite this result, spleen cells from these mice were still unable to proliferate in response to antigen after 3 days in culture.

Furthermore, when the antigen-stimulated cultures were continued for a further 24 hours, the S.I. of cells from mice infected with  $10^5$  mf was  $<1$  i.e. cpm in antigen-stimulated cells  $<$  cpm in cells in medium alone. In repeated experiments, the S.I. of spleen cells from mice infected with  $10^5$  mf in response to antigen after 4 days in culture was consistently  $<1$ , and was as low as 0.02. One explanation for this response is that the antigen preparation is toxic after prolonged culture. However, this is unlikely since neither spleen cells from L3-infected mice or from uninfected controls displayed a S.I. of  $<1$  in response to antigen after four days in culture. Alternatively, it could reflect antigen exhaustion after extended culture in the presence of antigen. However, this is also unlikely since L3-infected mice are the group that respond most vigorously to antigen at day three. Despite a significant decrease in the S.I. by day four in culture, spleen cells from L3-infected mice still proliferate significantly in response to antigen. A further possibility is that spleen cells from mf infected mice are undergoing some

form of antigen-induced cell death. The mechanism underlying the antigen-specific proliferative defect in mf-infected mice is further investigated in Chapter 6.

## **CHAPTER 4**

## Chapter 4 Analysis of the mechanisms underlying the down-regulation of polyclonal Th1 responses in L3-infected mice.

### 4.1. Introduction

The mechanisms through which Th polarized responses evolve and are maintained are likely to be both diverse and complex. Secretion of cross-inhibitory cytokines by Th cells themselves, in particular IL-4 and IL-10 by Th2 cells and IFN- $\gamma$  by Th1 cells, inhibit the activity of the opposing subset while promoting their own production. Therefore, once selected Th responses can become rapidly polarized (Mosmann and Coffman, 1989; Reiner and Locksley, 1993; Seder and Paul, 1994). A variety of other cell types including APC, NK cells, B cells and mast cells/basophils also produce cytokines that can influence Th activity (D'Andrea *et al*, 1992; Scott *et al*, 1993; Hsieh *et al*, 1993; Gazzinelli *et al*, 1993; Paul *et al*, 1993; Velupollai and Harn, 1994). A further level of Th cell control can be exerted by APC functions that are also influenced by cytokines (Murphy *et al*, 1994; Kubin *et al*, 1994; Cua *et al*, 1996).

The aim of the experiments in this chapter was to investigate the mechanisms by which infection with L3 down-regulates mitogen-stimulated Th1 responsiveness. The results in Chapter 3 provided some clues as to the mechanisms that might be involved. At day 12 p.i. the response is clearly Th2 biased, and the Th1 inhibitory cytokines, IL-4 and IL-10, are released only by splenocytes from L3-infected mice. In addition, the polyclonal Th1 defect was only apparent when L3 were given s.c., suggesting a role for APCs.

IL-4 can inhibit IFN- $\gamma$  production by mitogen-stimulated human PBMCs (Peleman *et al*, 1989). IL-4 by itself, or acting synergistically with IL-10, has been shown to suppress DTH (Powrie *et al*, 1993) and anti-*Leishmania* Th1 responses *in vivo* (Powrie *et al*, 1994). Furthermore, treatment of BALB/c mice with anti-IL-4 at the time of infection with *Trichuris muris* switches the subsequent response from Th2 to Th1 (Else

*et al*, 1994). In addition, *in vitro* culture with IL-4 can convert a *Leishmania*-specific Th1 population from a resistant mouse strain (C57BL/6) into a Th2 cytokine-expressing population that was stable and retained its phenotype *in vivo* when transferred into C57BL/6-SCID mice which were subsequently infected with *L. major* (Mocci and Coffman, 1995). This conversion occurred even when IL-4 KO mice were used as a source of *Leishmania*-specific Th1 cells, thus ruling out the possible outgrowth of an undetected Th2 population.

Treatment of mice with rIL-12 inhibits Th2 cytokine responses and stimulates antigen-specific IFN- $\gamma$  production induced by eggs of *S. mansoni* (Oswald *et al*, 1994) and mf of *B. malayi* (Pearlman *et al*, 1995). In addition, Th1 clones can be isolated by limiting dilution from *N. brasiliensis* infected mice in which a Th2 response dominates. Therefore, Th1 cells are thought to co-exist at low levels in Th2 polarized responses.

IL-10 inhibits mitogen-driven T cell proliferation and IL-2 and IFN- $\gamma$  production, acting primarily at the level of monocyte/macrophage accessory cells (Fiorentino *et al*, 1991; Ding and Shevach, 1992). IL-10 is known to downregulate MHC Class II expression (de Waal Malefyt *et al*, 1991), B7-2 costimulatory molecules (Murphy *et al*, 1994) and IL-12 production (D'Andrea *et al*, 1993) by accessory cells. These actions may culminate in the selective induction of anergy in the Th1 subpopulation (Villanueva *et al*, 1993), an effect that can be reversed by the addition of freshly isolated (non-IL-10 exposed) APC.

In mice infected with egg-producing *S. mansoni* antigen- and ConA-stimulated IL-2 and IFN- $\gamma$  production is reduced, coincident with the activation of a Th2 response (Pearce *et al*, 1991). *In vitro* treatment with anti-IL-10 caused a dramatic increase in antigen- and ConA-stimulated IFN- $\gamma$  production by spleen cells from mice with patent infections, indicating a functional role for IL-10 in Th1 downregulation *in vivo* in this model (Sher *et al*, 1991). The importance of IL-10 in cross-regulation *in vivo* is supported by studies

in *N. brasiliensis* infected IL-10 KO mice which develop elevated Th1 responses (Kuhn *et al*, 1993). Therefore, in normal mice IL-10 appears to limit the development of Th1 cells during a parasite-induced Th2 response *in vivo*.

In leprosy, IL-10 and IL-4 mRNA are expressed preferentially in lesions from lepromatous patients who exhibit reduced CMI. When PBMCs from lepromatous patients are cultured with anti-IL-10 or anti-IL-4, proliferative responses are enhanced (Sieling *et al*, 1993). Similarly, anti-IL-10 increases the antigen-stimulated proliferation of PBMCs from visceral leishmaniasis patients with impaired CMI responses (Ghalib *et al*, 1993). In filariasis, IL-10 has also been implicated in inhibiting parasite-specific Th1 responses. Treatment of PBMCs from hyporesponsive microfilaraemics with anti-IL-10 enhanced their antigen-specific proliferative responses (King *et al*, 1993). Mice chronically infected with live mf or multiply immunised with mf extract generate Th2 responses, characterised by the secretion of antigen-specific IL-4 and IL-5 by CD4<sup>+</sup> spleen and lymph node cells (Pearlman *et al*, 1993). Addition of neutralizing anti-IL-10 to these cultures significantly increased antigen-driven IFN- $\gamma$  production.

Severely impaired splenocyte proliferation and IL-2 production in response to mitogen is not uncommon following infection with a variety of intracellular protozoa (Harel-Bellan *et al*, 1983; Silva *et al*, 1992; Haque *et al*, 1994; Candolfi *et al*, 1994), bacteria (Gercken *et al*, 1994; Gregory *et al*, 1993) and viruses (Saron *et al*, 1990). In such cases the impairment tends to be general and can extend to B cell function and, as such, is different from the situation following infection with L3 in which there is a specific Th1 defect. However, there may be some overlap in underlying mechanisms. Defective antigen presentation (Gercken *et al*, 1994), and the production of NO (Gregory *et al*, 1993; Candolfi *et al*, 1994) and IL-10 (Silva *et al*, 1992; Haque *et al*, 1994) by APCs have all been implicated in the immunosuppressive effects of intracellular parasitic infection. In addition, insufficient costimulation by APC can result in T cell unresponsiveness (Jenkins and Schwartz, 1987).

Therefore, in this chapter attempts were made to restore defective polyclonal Th1 responses *in vitro* using anti-IL-4, anti-IL-10, rIL-2 and by replacement of the adherent cell population. While the focus of the experiments was on the impaired Th1 responses to mitogen, infection with L3 also results in antigen-specific Th1 non-responsiveness. Although spleen cells from L3-infected mice proliferate in response to antigen, only Th2 cytokines were secreted and neither IL-2 nor IFN- $\gamma$  was detectable. Therefore, the *in vitro* manipulations described above were also performed on antigen-stimulated spleen cell cultures to determine whether antigen-specific Th1 cells are absent, or are primed but suppressed, during the L3-induced Th2 response.

## **4.2. Results**

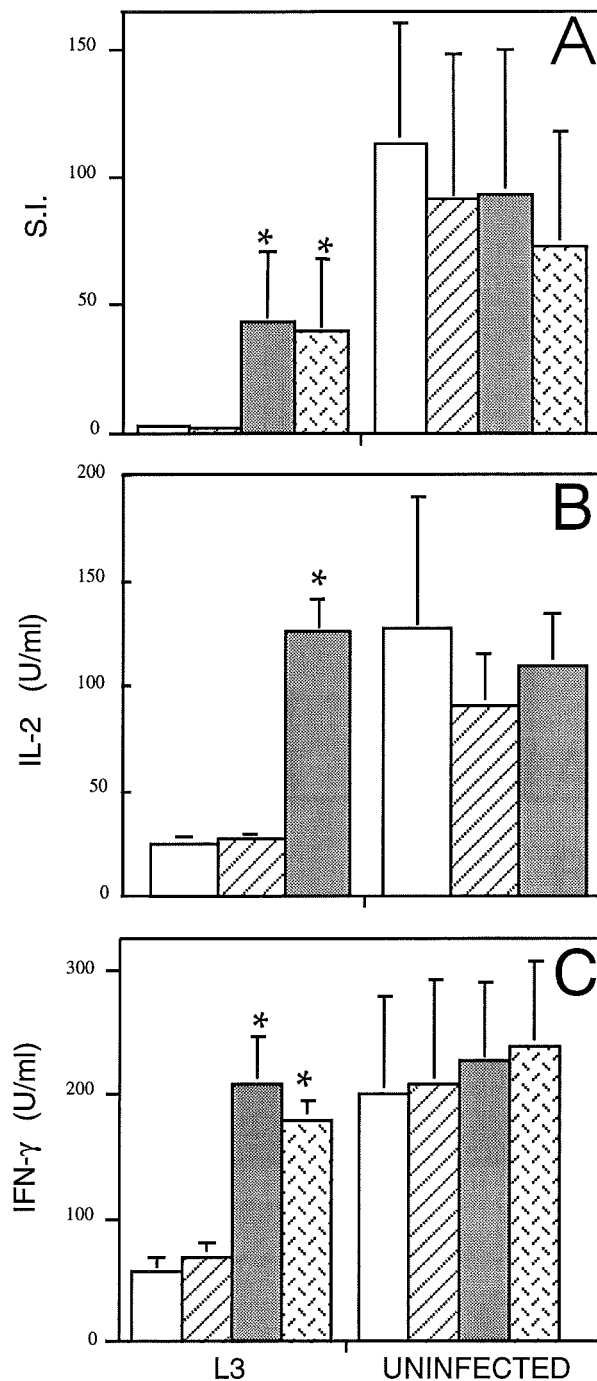
### **4.2.1. *In vitro* treatment with anti-IL-4 or rIL-2 partially restores the defective mitogen-driven Th1 responses of spleen cells from mice infected with L3**

The results of Chapter 3 suggest a role for IL-4 and/or a lack of IL-2 in the impaired polyclonal responses in L3 infected mice. To examine these possibilities, 11B11, a neutralizing anti-IL-4 MAb, or rIL-2 was added to ConA-stimulated spleen cell cultures from mice infected with either L3 or from uninfected controls, and proliferation and cytokine responses monitored. Anti-IL-4 treatment caused a dramatic enhancement of ConA-induced proliferation (S.I. L3 infected mice plus 11B11=42.9), although responses were not restored to the level seen in uninfected mice (S.I.=113.0,  $p = 0.016$ ) (Fig. 4.1A). In contrast, anti-IL-4 completely restored IL-2 and IFN- $\gamma$  levels to those of uninfected controls with no treatment ( $p > 0.05$  for both cytokines, Fig. 4.1B & C) while having no effect on mitogen-induced IL-5 or IL-10 production (data not shown). Exogenous IL-2 increased ConA-stimulated IFN- $\gamma$  production to levels equivalent to spleen cells from uninfected controls with no treatment ( $p > 0.05$ ) (Fig. 4.1C), but, as with anti-IL-4, the Con-A induced proliferative response was only partially restored ( $p = 0.008$ ) (Fig. 4.1A).

### **4.2.2. Antigen-stimulated proliferation following infection with L3 can be substantially blocked *in vitro* by anti-IL-4**

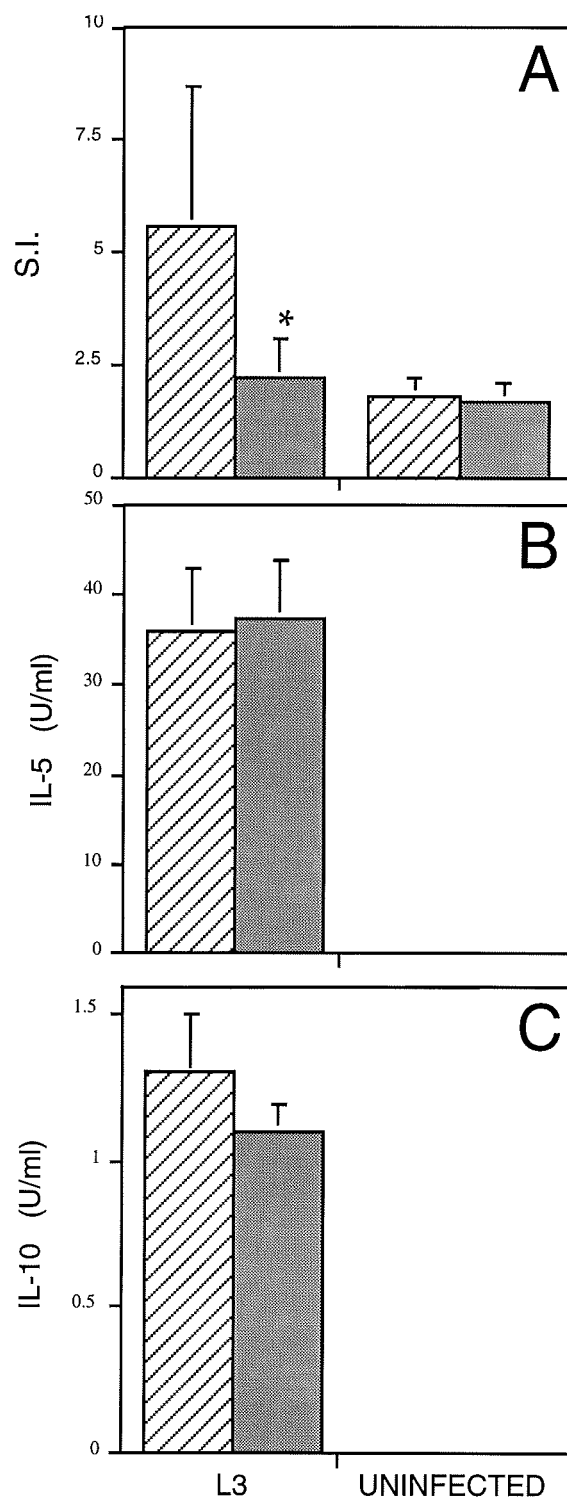
The ability of spleen cells from mice infected with L3 to proliferate in response to specific antigen yet produce only Th2 cytokines implies that the proliferation detected is due to Th2 cell expansion. Spleen cells from L3-infected or uninfected mice were cultured in the presence of antigen plus 11B11 or isotype-matched control and the effect on proliferation and cytokine production was measured. Figure 4.2A shows that treatment with anti-IL-4 significantly reduces antigen-specific proliferation by spleen cells from mice given L3, if compared to isotype matched control ( $p = 0.008$ ).







**FIGURE 4.1. The effect of neutralizing anti-IL-4 MAb on ConA-driven responses of spleen cells from mice infected with L3**

Mice were injected s.c. with 50 L3 or HBSS and spleens removed at day 12 p.i. ConA-stimulated cultures were incubated alone□, or with 10 $\mu$ g/ml of either an isotype matched control (R59-40)▨ or anti-IL-4 (11B11)■, or 100 U/ml rIL-2▩. After 48 h incubation, proliferation (A) and IL-2 (B) and IFN- $\gamma$  (C) production were measured, as described previously. The results shown are the mean  $\pm$  SD of 5 animals per group. \* Significant difference ( $p < 0.05$ ) between value obtained with 11B11 and with isotype matched control MAb or between value obtained with rIL-2 and with no treatment control. S.I.'s were also significantly different ( $p < 0.05$ ) between the cells of L3 infected mice treated with 11B11 or rIL-2 and uninfected mice with no treatment.



**FIGURE 4.2. The effect of neutralizing anti-IL-4 MAb on antigen-driven responses of spleen cells from mice infected with L3.**

Mice were injected as described in the legend to Fig. 4.1. Antigen-stimulated cultures were incubated with 10 µg/ml of either an isotype matched control (R59-40)  or anti-IL4 (11B11)  and proliferation (A), and IL-5 (B) and IL-10 (C) production were measured, as described previously. The results shown are the mean ± SD of 5 animals per group. \* Significant difference ( $p < 0.05$ ) from value obtained without 11B11.

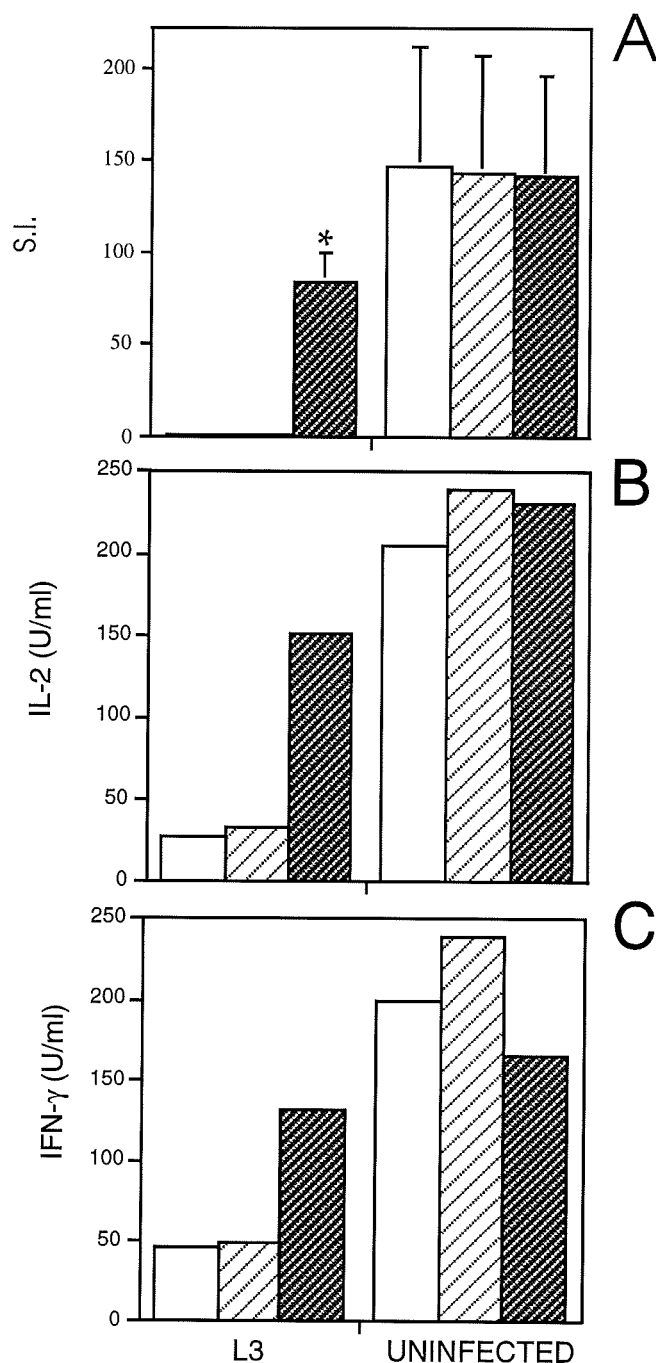
However, anti-IL-4 treatment did not result in any IL-2 or IFN- $\gamma$  production (data not shown), nor did it affect antigen-driven IL-5 or IL-10 production by spleen cells from these mice ( $p > 0.05$ )(Fig. 4.2B & C).

#### **4.2.3. *In vitro* treatment with anti-IL-10 partially restores the defective mitogen-driven Th1 responses of spleen cells from mice infected with L3**

The addition of anti-IL-4 or rIL-2 to spleen cell cultures from L3-infected mice failed to fully restore the ConA-stimulated proliferative response, or to stimulate antigen-specific Th1 responses. Therefore, further experiments were performed to examine the role of IL-10 in generating defective Th1 responses. A neutralizing anti-IL-10 MAb (JESS) was added to ConA-stimulated spleen cell cultures from mice infected with either L3 or from uninfected controls and the proliferation and cytokine responses measured. Fig. 4.3A shows that anti-IL-10 treatment resulted in a significant increase in the ConA-induced proliferative response (SI for L3-infected mice plus JESS= 84.6), but not to the level of uninfected mice (SI=146.9,  $p = 0.048$ ). Since a limited amount of anti-IL-10 MAb was available, the cytokine assays were performed using spleen cells pooled from 5 animals in each group. The experiment was performed three times with equivalent results. Anti-IL-10 treatment dramatically enhanced ConA-stimulated IL-2 and IFN- $\gamma$  secretion (Fig. 4.3B and C), but had no effect on IL-4 or IL-5 production by spleen cells from L3-infected mice (data not shown).

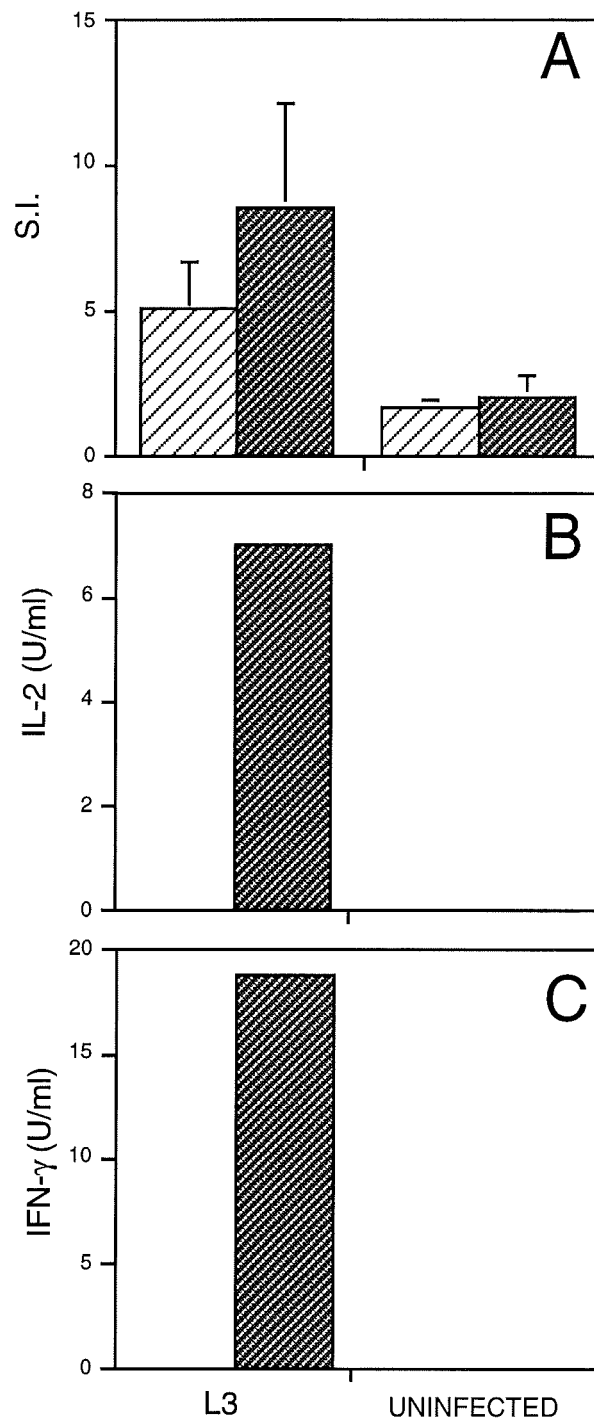
#### **4.2.4. *In vitro* treatment with anti-IL-10 stimulates antigen-specific Th1 responses by spleen cells from mice infected with L3**

Neutralizing anti-IL-10 MAb (JESS) was also added to antigen-stimulated spleen cell cultures from mice infected with either L3 or from uninfected controls and the proliferation and cytokine responses measured. Anti-IL-10 had no effect on antigen-stimulated proliferation (Fig. 4.4A) or on IL-4 and IL-5 secretion (data not shown) of spleen cells from L3-infected mice. However, anti-IL-10 did stimulate the secretion of antigen-driven IL-2 and IFN- $\gamma$  by spleen cells from L3-infected mice (Fig. 4.4B and C).





**FIGURE 4.3. The effect of neutralizing anti-IL-10 MAb on ConA-driven responses of spleen cells from mice infected with L3.**

Mice were injected as described in the legend to Fig. 4.1. ConA-stimulated cultures were incubated alone □, or with 10 µg/ml of either an isotype matched control (R59-40) ▨ or anti-IL-10 (JESS) ■. After 48 h incubation, proliferation (A) and IL-2 (B) and IFN-γ (C) production were measured, as described previously. (A) The S.I.s are the mean ± SD of 5 animals per group. \* Significant difference ( $p < 0.05$ ) between value obtained with JESS and with isotype matched control MAb. S.I.'s were also significantly different ( $p < 0.05$ ) between the cells of L3 infected mice treated with JESS and uninfected mice with no treatment. (B) & (C) Cytokine assays were performed on spleen cells pooled from 5 animals in each group. The results presented were comparable in two additional experiments.



**FIGURE 4.4. The effect of neutralizing anti-IL-10 MAb on antigen-driven responses of spleen cells from mice infected with L3.**

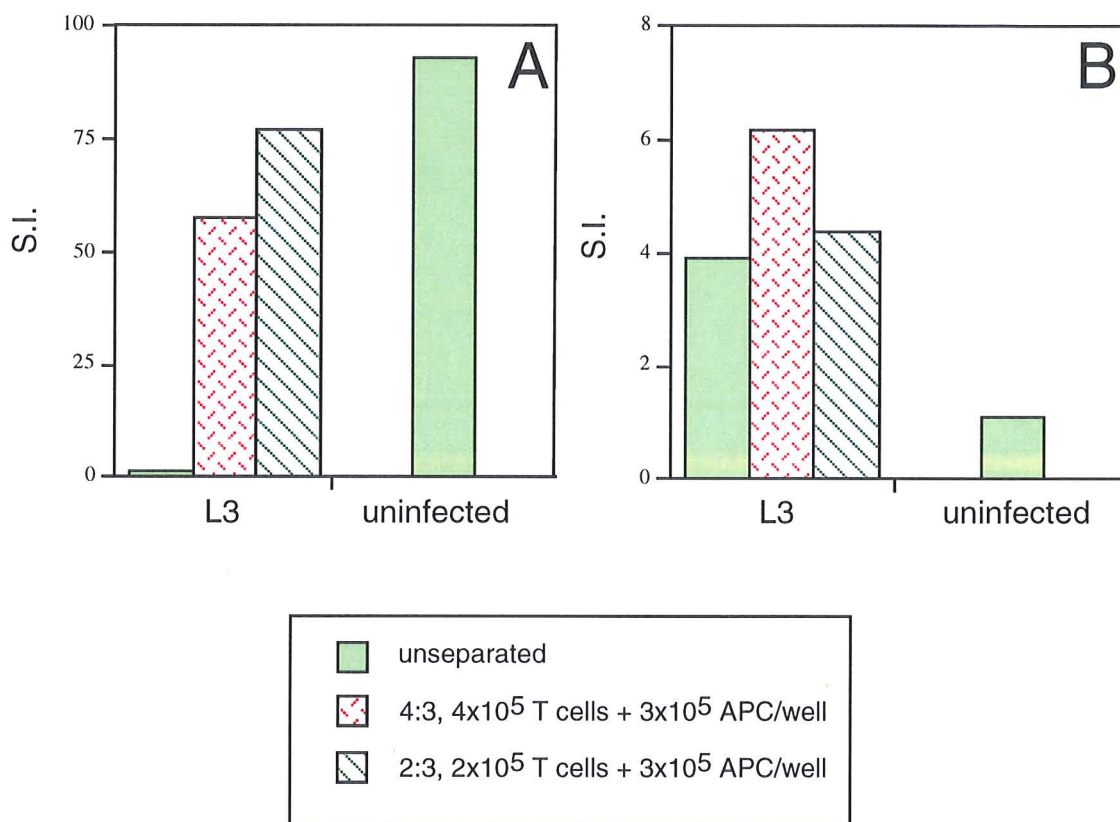
Mice were injected as described in the legend to Fig. 4.1. Antigen-stimulated cultures were incubated with 10 µg/ml of either an isotype matched control (R59-40)  or anti-IL-10 (JESS)  and proliferation (A), and IL-2 (B) and IFN-γ (C) production were measured, as described previously. (A) The S.I.s shown are the mean ± SD of 5 animals per group. \* Significant difference ( $p < 0.05$ ) from value obtained without JESS. (B) & (C) Cytokine assays were performed on spleen cells pooled from five animals in each group. The results presented were comparable in two additional experiments.

#### **4.2.5. Depletion of nylon wool adherent cells from spleens of L3-infected mice dramatically enhances mitogen-driven Th1 responses**

This experiment was conducted to assess the role of APC (nylon wool adherent cells in the spleen) in down-regulating mitogen Th1 responses. Splenic T cells pooled from five L3-infected mice were purified over nylon wool columns and incubated with irradiated, syngeneic spleen cells from uninfected mice. The ratio of T cell:APC used were 2:3 and 4:3 for the proliferation assays and 2:3, 4:3 and 3:2 for the cytokine assays. In the proliferation assays irradiated spleen cells from L3-infected mice were also used as a source of APC.

In three separate experiments, depletion of nylon wool adherent cells resulted in a dramatic increase in polyclonal proliferation relative to unseparated spleen cells from the same animals and to a level comparable to that of spleen cells from uninfected animals. The results of a representative experiment are shown in Fig. 4.5A. This effect was independent of the source of the APC population. Irradiated spleen cells from uninfected or infected mice both restored the defective T cell proliferative response to mitogen (data not shown). Furthermore, in this experiment, depletion of nylon wool adherent cells restored ConA-driven IL-2 and IFN- $\gamma$  to the level of uninfected animals (Fig. 4.6A & B).

The effect on secretion of Th2 cytokines varied between three experiments, depending on the extent of the recovery in Th1 cytokines production. In the first experiment production of IL-4, IL-5 and IL-10 (Fig. 4.6C, D & E) was reduced to below background and IL-2 and IFN- $\gamma$  were fully recovered to the level of uninfected animals. In the second experiment (one T cell:APC ratio was used; 3:2), IL-4 and IL-10 production were reduced to below background, but there was no effect on IL5 production, and IL-2 and IFN- $\gamma$  were only partially recovered (Fig. 4.7). In the third experiment (all three T cell:APC ratios were used; 2:3, 4:3 and 3:2), there was no effect



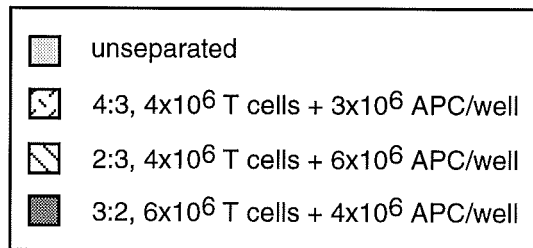
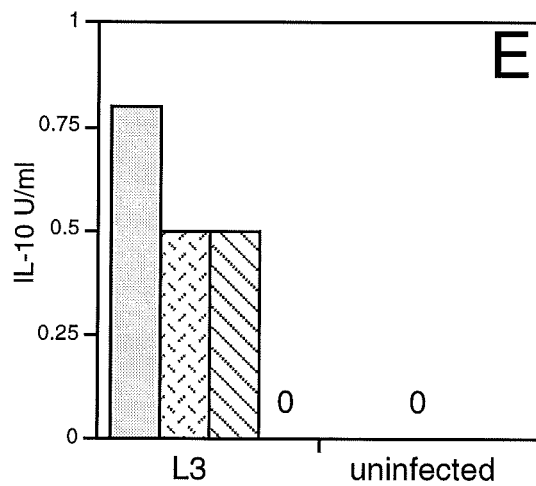
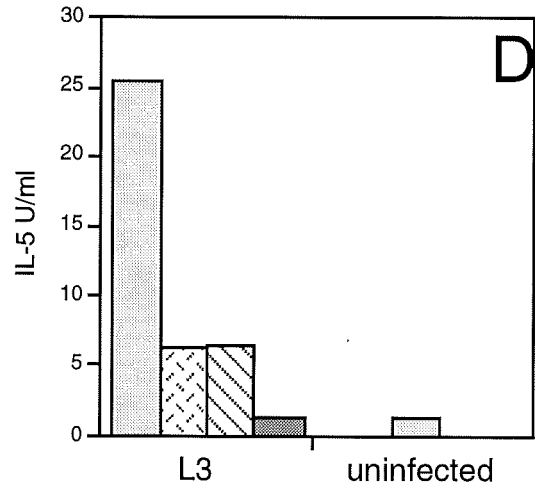
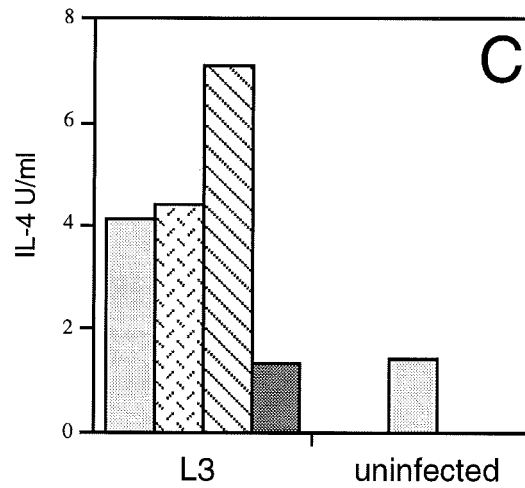
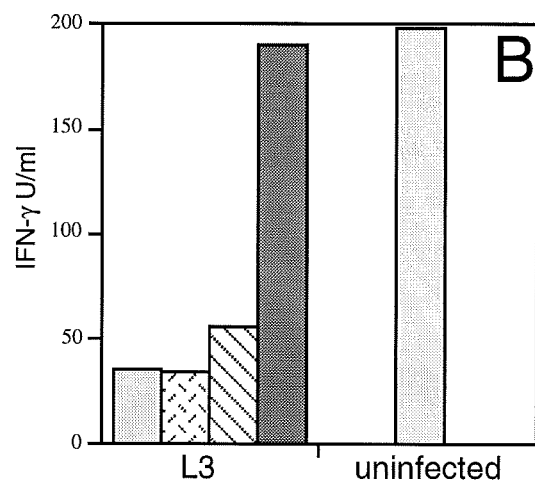
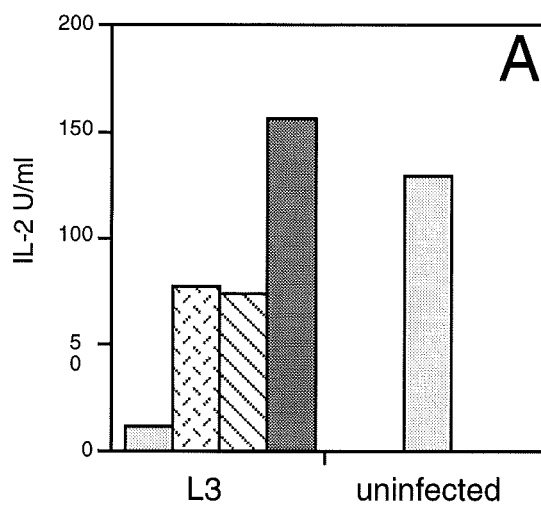
**FIGURE 4.5. The effect of depletion of nylon wool adherent cells from spleens of L3-infected mice on mitogen and antigen driven proliferation.**

Mice were injected as described in the legend to Fig. 4.1. Spleen cells from 5 mice in each group were pooled. Splenic T cells from L3-infected mice, purified by passing the pooled spleen cell suspension over nylon wool columns, were incubated with irradiated, syngeneic spleen cells from uninfected mice. The ratios of T cell: APC used for proliferation assays were 2:3 and 4:3 (total cell number per well =  $5 \times 10^6$ ). (A) ConA- and (B) antigen-stimulated proliferation were measured in these cultures and in cultures of unseparated spleen cells from L3-infected and uninfected mice. The cytokine responses in this experiment are presented in Figs 4.6 (ConA) & 4.9 (antigen)

**FIGURE 4.6. The effect of depletion of nylon wool adherent cells from spleens of L3-infected mice on mitogen-driven cytokine responses I**

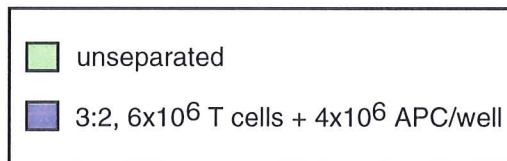
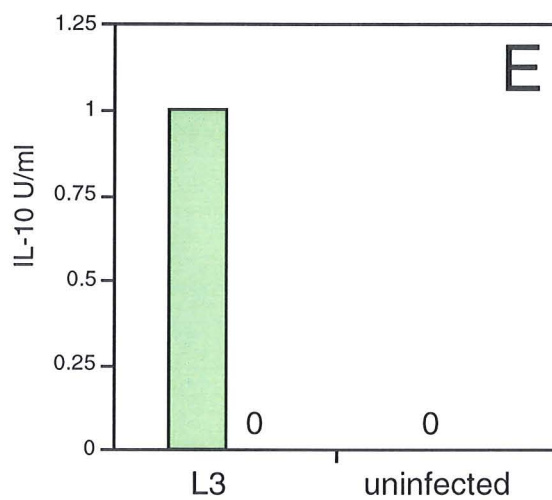
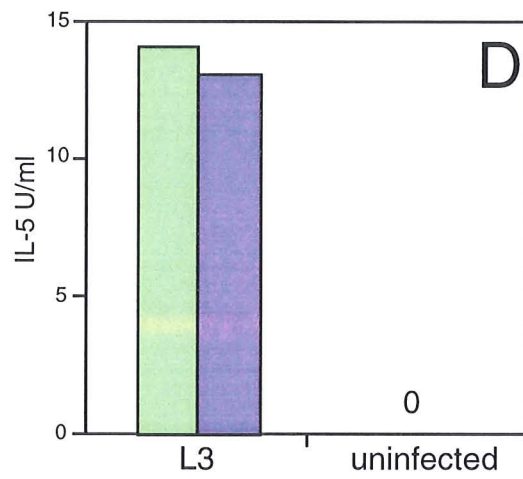
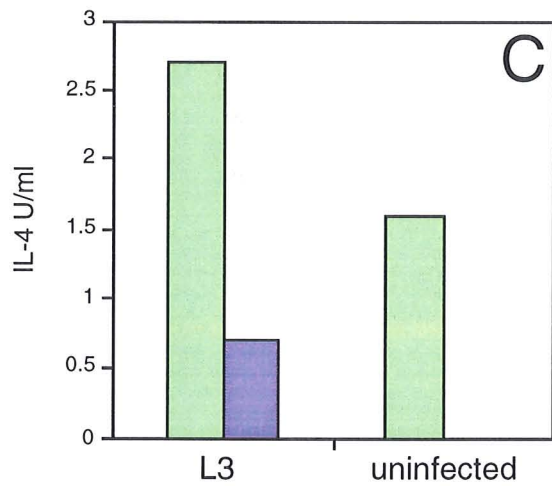
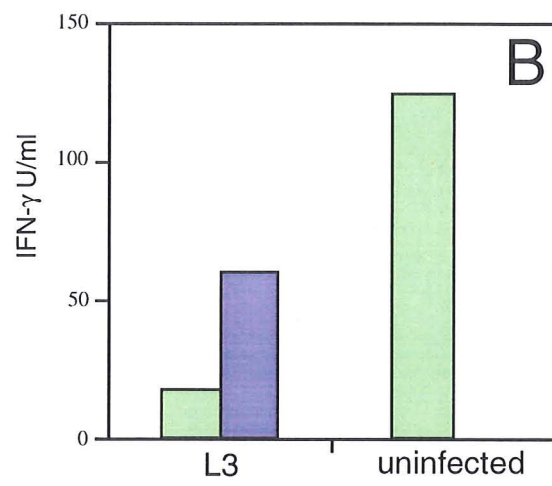
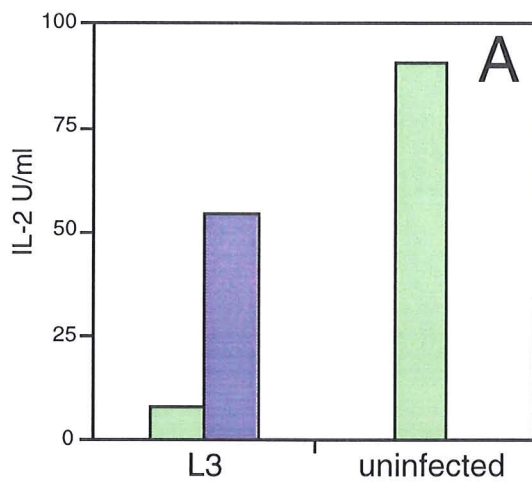
Mice were injected as described in the legend to Fig. 4.1. Spleen cells were depleted as described in the legend to Fig. 4.5. The ratios of T cell: APC used for cytokine assays were 4:3, 2:3 and 3:2 (total cell number per well= $1 \times 10^7$ ). After 48 h incubation, ConA-stimulated IL-2 (A), IFN- $\gamma$  (B), IL-4 (C), IL-5 (D) and IL-10 (E) production were measured in these cultures and in cultures of unseparated spleen cells from L3-infected and uninfected mice. A value of 0 indicates a reading below the sensitivity of the assay. The results of ConA-stimulated cytokine production in two additional experiments are presented in Figs 4.7 and 4.8.





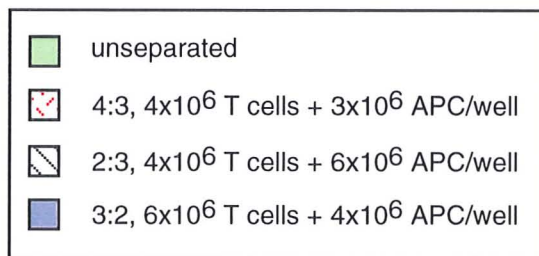
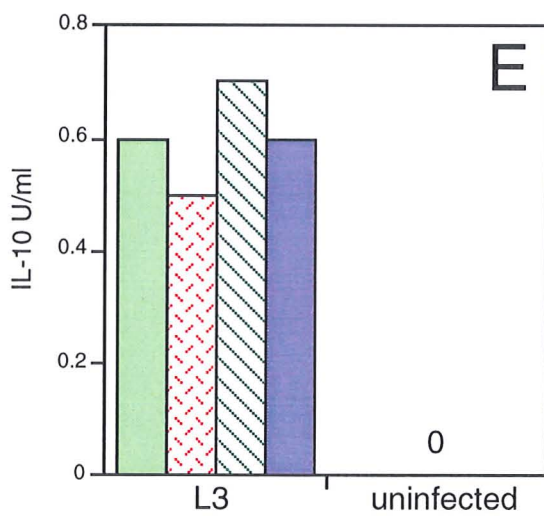
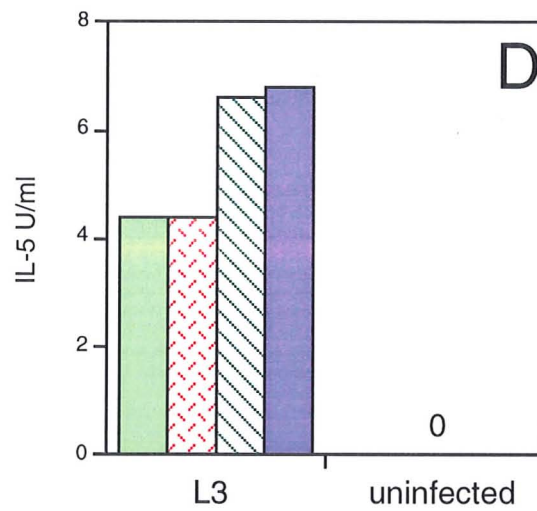
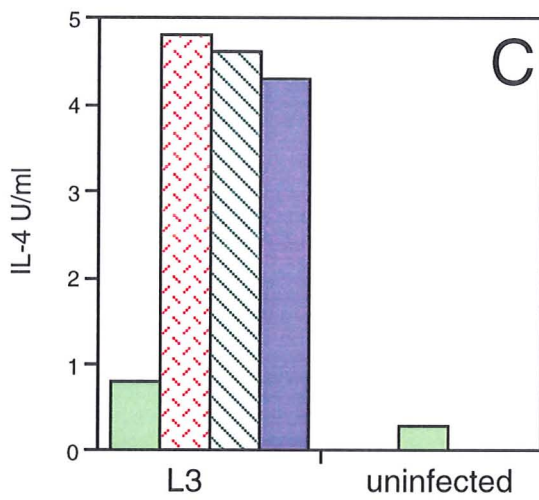
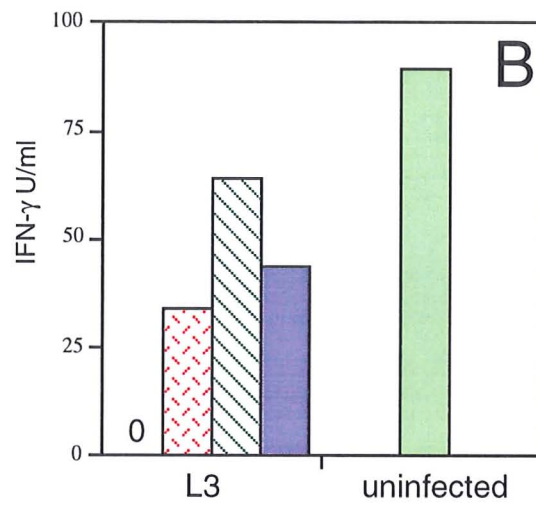
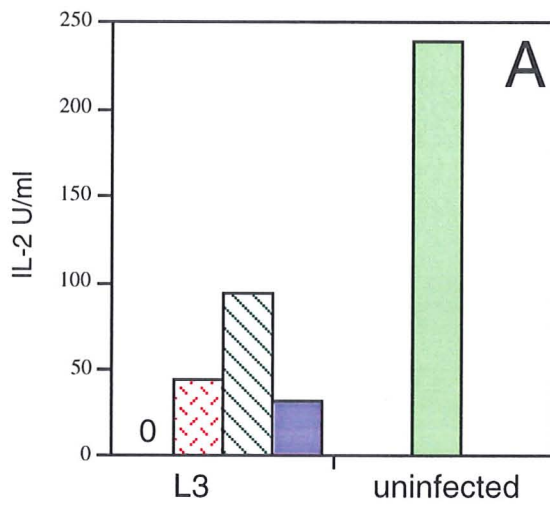
**FIGURE 4.7. The effect of depletion of nylon wool adherent cells from spleens of L3-infected mice on mitogen-driven cytokine responses II**

Mice were injected as described in the legend to Fig. 4.1. Spleen cells were treated as described in the legend to Fig. 4.6, except that only one T cell: APC ratio was analysed, 3:2 (total cell number per well= $1 \times 10^7$ ). ConA-stimulated IL-2 (A), IFN- $\gamma$  (B), IL-4 (C), IL-5 (D) and IL-10 (E) production is presented above. A value of 0 indicates a reading below the sensitivity of the assay.



**FIGURE 4.8. The effect of depletion of nylon wool adherent cells from spleens of L3-infected mice on mitogen-driven cytokine responses III**

Mice were injected as described in the legend to Fig. 4.1.. Spleen cells were treated as described in the legend to Fig. 4.6. ConA-stimulated IL-2 (A), IFN- $\gamma$  (B), IL-4 (C), IL-5 (D) and IL-10 (E) production is presented above. A value of 0 indicates a reading below the sensitivity of the assay.



on IL-4, IL-5 or IL-10 production, and IL-2 and IFN- $\gamma$  was only partially recovered (Fig. 4.8).

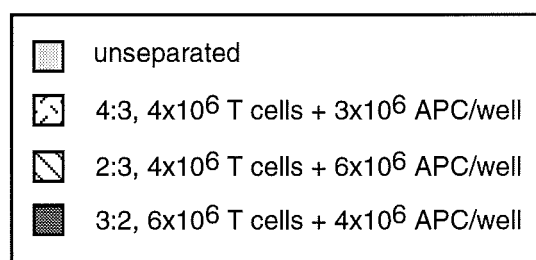
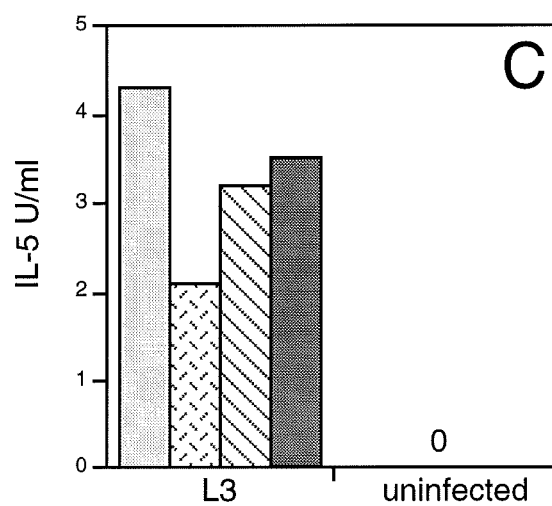
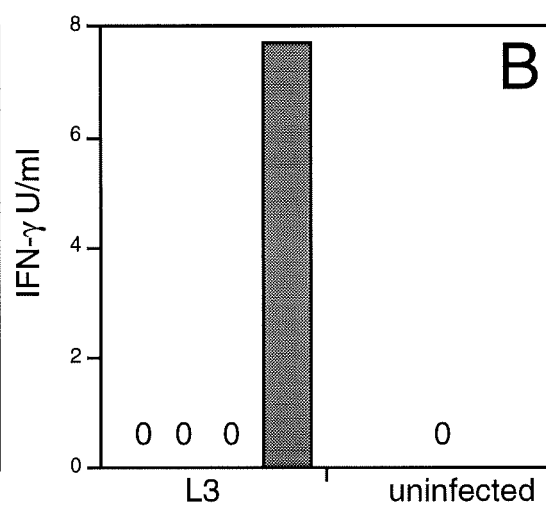
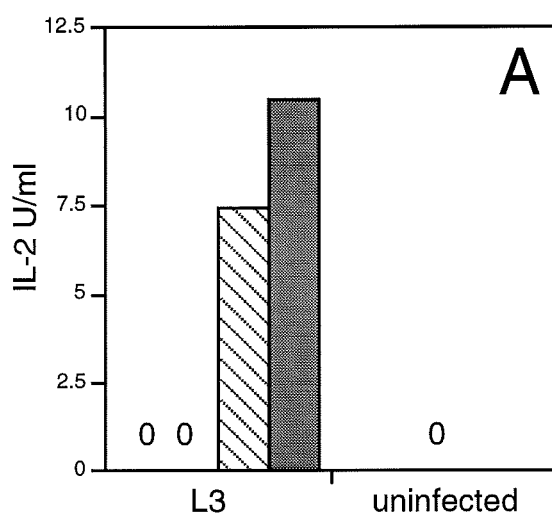
#### **4.2.6. Depletion of nylon wool adherent cells from spleens of L3-infected mice stimulates antigen-specific Th1 responses**

The role of APC in suppressing antigen-specific Th1 responses was then assessed. The ratios of T:APC used were the same as for the analysis of mitogen-driven responses. In the three separate experiments, both irradiated APC from uninfected and L3-infected mice were able to stimulate antigen-specific proliferation of T cells from L3-infected mice to levels comparable to that of unseparated spleen cells. The results of a representative experiment are shown in Fig. 4.5*B*. Therefore, irradiated APC populations are able to process and present antigen normally.

Depletion of nylon wool adherent cells also enabled T cells from L3-infected mice to produce antigen-specific IL-2 and IFN- $\gamma$  production (Fig. 4.9*A & B*). Unseparated spleen cells from L3 infected mice did not secrete IL-2 and IFN- $\gamma$  in responses to antigen. In this experiment, replacement of the APC population had no effect on IL-5 secretion (Fig. 4.9*C*). However, since no IL-4 or IL-10 could be detected in antigen-stimulated cultures of unseparated spleen cells from L3-infected mice, the influence of APC on the production of these cytokines could not be assessed.

**FIGURE 4.9. The effect of depletion of nylon wool adherent cells from spleens of L3-infected mice on antigen-driven cytokine responses**

Mice were injected as described in the legend to Fig. 4.1. Spleen cells were treated as described in the legend to Fig. 4.6. The ratios of T cell: APC used in proliferation and cytokine assays were the same as that described in the legend to Fig. 4.5. Ag-stimulated IL-2 (A), IFN- $\gamma$  (B) and IL-5 (C) production were measured in these cultures and in cultures of unseparated spleen cells from L3-infected and uninfected mice. A value of 0 indicates a reading below the sensitivity of the assay. The results presented are representative of two additional experiments.





### 4.3. Discussion

To determine the mechanism(s) underlying the profound Th cell imbalance following infection with L3, attempts were made to restore defective responses *in vitro*. The results of these experiments clearly demonstrate that IL-4, IL-10 and the APC population in infected mice, play a critical role in down-regulating polyclonal Th1 responses. In addition, IL-10, but not IL-4, appears to be the critical factor in suppressing antigen-specific Th1 activity.

When neutralizing anti-IL-4 or anti-IL-10 was added to ConA-stimulated spleen cells from mice infected with L3, IL-2 and IFN- $\gamma$  production were dramatically augmented while proliferation was partially restored. The addition of rIL-2 to the cultures also resulted in the recovery of proliferation and IFN- $\gamma$  production in response to ConA suggesting that Th1 down-regulation following infection with L3 may operate via a block on IL-2 production. However, anti-IL-4 treatment had no effect on mitogen-driven IL-10 (or IL-5) secretion. Similarly, anti-IL-10 treatment had no effect on mitogen-driven IL-4 (or IL-5) secretion. The presence of both IL-4 and IL-10 in the treated cultures, may explain the inability to fully recover the proliferative response using anti-IL-4 or anti-IL-10 alone. Alternatively the proliferative defect may be less easily reversed following short-term *in vitro* culture. Other studies have demonstrated that proliferation and cytokine production appear to be differentially regulated (Evavold and Allen, 1993; Allen *et al*, 1996). It would be interesting to investigate the mode of action of the L3-induced IL-4 and IL-10 in this system. Previously, IL-4 was thought to directly inhibit the activity of established Th1 cells (Peleman *et al*, 1989), while IL-10 is known to inhibit T cells indirectly by acting on accessory cells (Ding and Shevach, 1992). However, a recent study has shown that both IL-4 and IL-10 can directly affect the ability of a macrophage APC population to support the priming of Th1 cells (Cua *et al*, 1996). In addition, IL-10 can inhibit T cell proliferation and IL-2 production in the absence of APC (de Waal Malefyt *et al*, 1993). Furthermore, the source of L3-

stimulated IL-10 is not definite. IL-10 is produced primarily by Th2 cells, Ly-1 B cells, activated mast cell lines and activated macrophages (Moore *et al*, 1993). Since CD4<sup>+</sup> LN T cells are responsible for antigen-specific IL-10 following injection of L3 into the footpad (See 5.2.5), it is likely that IL-10 is produced predominantly by Th2 cells in the spleen. However, the possibility cannot be ruled out that APC-derived IL-10 acts during cognate T-APC interaction. APC-derived IL-10 has been shown to impair cellular responses in acute murine toxoplasmosis (Haque *et al*, 1994). The suppressive effect of adherent spleen cells from *T. gondi*-infected mice on ConA-induced IL-2 production by normal splenocytes was abrogated by the addition of anti-IL-10 to these cultures. A similar experiment could be attempted with adherent spleen cells from L3-infected mice.

The direct influence of APC in our system was also investigated. Mitogen-driven Th1 responses were impaired only when L3 were given s.c.; infection with L3 by the i.p. route was unable to down-regulate polyclonal Th1 responses in the spleen even when mitogen-stimulated IL-4 and IL-10 could be detected. When spleen cell preparations, from mice infected s.c. with L3, were depleted of nylon wool adherent cells and replaced with irradiated spleen cells from uninfected mice, proliferation and IL-2 and IFN- $\gamma$  production were restored to that of uninfected controls. In addition, Th2 cytokine production was reduced in the experiments where IL-2 and IFN- $\gamma$  production was almost or completely recovered. Therefore, s.c. infection with L3 would appear to influence the function of the APC population in the spleen. Various APC-derived factors in addition to IL-10 are known to suppress T cell proliferation including NO, PGE<sub>2</sub>, H<sub>2</sub>O<sub>2</sub> and TGF- $\beta$  (Haque *et al*, 1994; Albina *et al*, 1991; Metzger *et al*, 1980; Barral-Netto *et al*, 1992). The addition of known inhibitors or the appropriate neutralising antibodies would determine whether any of these mediators is operative in ConA-stimulated spleen cell cultures from L3-infected mice. In addition, the splenic APC population of infected mice could be fractionated more selectively to evaluate the role of different cell types. Con A-stimulated proliferation was recovered completely

when irradiated spleen cells from L3-infected mice were added back as a source of APC. Since B cells are more susceptible to the effect of irradiation than other APC types e.g. dendritic cells (Sopori *et al*, 1985), B cells may be the critical cell in inducing Th1 unresponsiveness to mitogen in this model.

A role for APC in the suppression of T cell proliferative responses during filarial infection has been reported in other studies. Jirds infected with *B. pahangi* exhibit unresponsiveness to mitogen, that could be restored by removal of adherent spleen cells (Portaro *et al*, 1976; Lammie and Katz, 1983). In addition, spleen cells from infected jirds that had been pre-exposed to ConA or *Brugia* antigen *in vitro* induced unresponsiveness to T and B cell mitogens in co-cultured normal lymphocytes. This suppression was not mediated by spleen cells from uninfected animals and again could be reversed by removal of plastic-adherent cells (Lammie and Katz, 1984). The adherent cell populations from infected jirds may release suppressive factors or be unable to process or present antigen efficiently. However, in the absence of cell-surface phenotypic markers and other immunological reagents for the jird, the nature of the adherent cell populations or the mechanism(s) by which they are able to exert specific and non-specific T and B cell suppression could not be further investigated.

More recently, it has been shown that adherent peritoneal exudate cells (PEC), from mice implanted i.p. with *B. malayi* adult worms, blocked antigen-specific proliferation of a conalbumin-specific Th2 cell clone (D10G.4) (Allen *et al*, 1996). PEC from infected mice were able to process and present conalbumin normally since the D10 cell line secreted IL-4. In addition, the suppressive effect on T cell proliferation was not due to an absence of costimulation since it was overcome when infection-derived PEC were diluted in the presence of fixed numbers of mitomycin C-treated splenocytes. Uncoupling of proliferative and cytokine responses has been reported previously using altered peptide ligands (APL) (Evavold and Allen, 1991). Interestingly, APL can also induce T cell anergy (Sloan-Lancaster *et al*, 1993) which is thought to occur from TCR

occupancy in the absence of proliferation (DeSilva *et al*, 1991). Therefore, the proliferative block on parasite-specific cells at the site of infection may represent some form of T cell anergy. The addition of indomethacin (inhibitor of PGs), catalase (inhibitor of H<sub>2</sub>O<sub>2</sub>), L-NMMA (inhibitor of NO) or anti-TGF- $\beta$  to the cultures had no effect on T cell proliferation and the mediator of the suppression remains to be determined. In contrast, further experiments demonstrated that PEC from mf-infected mice also blocked proliferation of D10G.4 T cells that could be overcome by inhibition of NO production using L-NMMA. A different mechanism of T cell suppression is consistent with the contrasting cytokine responses elicited by adults and mf. IFN- $\gamma$  produced exclusively in response to mf is known to be a potent inducer of NO (Ding *et al*, 1988), whereas adults, that stimulate predominantly IL-4 (Lawrence *et al*, 1994) induce a suppression that is not mediated by NO. Furthermore, IL-4 is known to inhibit NO (Al-Ramadi *et al*, 1992) and the regulation of NO production by IL-4 and IFN- $\gamma$  has been suggested as a novel pathway of Th regulation via the macrophage (Liew *et al*, 1991). Since L3 also induce IL-4 rather than IFN- $\gamma$  production, it seems unlikely that the mechanism of mitogen-driven Th1 unresponsiveness following infection with L3 is NO dependent. However, it would be necessary to treat spleen cells with L-NMMA to rule this out.

APC-surface molecules may be also responsible for selectively inhibiting Th1 activity. APC costimulatory molecules, like B7-1 and B7-2, mediate costimulation and prevent the induction of T cell anergy via their interaction with CD28 (Lenshow *et al*, 1996). However, the effect of APC costimulation on Th1/Th2 cell biology is controversial. On one hand, anergy appears to be more easily induced in Th1 than Th2 effector cells (Williams *et al*, 1990; Burstein *et al*, 1992), so that under certain circumstances Th1 cells can become anergised while Th2 cells are activated (De Wit *et al*, 1992; Peterson *et al*, 1993). For example, the stimulation of Th0 lines in the absence of appropriate co-stimulation results in the selective loss of Th1 characteristics and the retention of a Th2 phenotype (Gajewski *et al*, 1994). In addition, IL-10 is known to down-regulate B7-2

expression (Murphy *et al*, 1994) resulting in Th1 anergy (Ding and Shevach, 1992; Villanueva *et al*, 1993). However, other studies using naive T cells demonstrate that in the absence of CD28 signalling Th1 responses dominate while CD28 co-stimulation is necessary for Th2 responses (Lenschow *et al*, 1996). For example, blocking CD28/B7 interaction using hCTLA4-Ig blocked the induction of a susceptible Th2 response in BALB/c mice infected with *L. major* but had no effect in the resistant Th1-type C57BL/6 mouse strain (Corry *et al*, 1994). In addition, IL-4 upregulates B7-2, and to a lesser extent B7-1, on splenic B cells (Stack 1994). However, these differences may reflect the changes in relative expression of costimulatory molecules and their positive and negative signalling activities during the induction, ongoing and end phases of an immune response (Lenschow *et al*, 1996). A role for the CD28/B7 interaction in the down-regulation of Th1 responses could be assessed by incubating spleen cells from L3-infected mice with an anti-CD28 MAb that can replace APC co-stimulatory function or alternatively blocking the interaction with CTLA4-Ig.

In addition, it cannot be ruled out that molecules released by the L3 may be directly involved in down-regulating T cell activity. Filarial E-S products have been implicated in immunosuppression in filariasis in both human and animal models. Serum from infected patients or animals is able to interfere with lymphocyte proliferation *in vitro* (Piessens *et al*, 1980a; Lammie *et al*, 1984). High molecular weight components of microfilariae (Leiva and Lammie, 1989) and PC-containing antigens of adult *B. malayi* (Lal *et al*, 1990) can inhibit mitogen induced lymphocyte proliferation. ES-62, a PC-bearing antigen of the filarial parasite *Acanthocheilonema viteae*, has been shown to inhibit polyclonal activation of B cells, by interfering with the Ag receptor signalling pathway (Harnett and Harnett, 1993). In addition, filarial parasites may produce prostaglandins that are known to have T cell suppressive activity (Liu 1992).

*In vitro* treatment with anti-IL-4 also substantially reduced antigen-specific proliferation, suggesting that the proliferation detected reflects Th2 expansion.

However, in the presence of 11B11 or there was no coincident production of antigen-specific IL-2 or IFN- $\gamma$  demonstrating that, if antigen-primed Th1 cells are present, they are still unable to secrete Th1 cytokines in the absence of IL-4. A further interpretation of this data is that primary infection with L3 of *Brugia* does not result in any Th1 priming *in vivo*. That this may indeed be the case is supported by experiments in which IL-4 KO mice infected with the L3 of *Brugia* failed to produce IFN- $\gamma$ , even in the absence of IL-4 *in vivo* (Lawrence *et al*, 1995). However, treatment with anti-IL-10 did stimulate the secretion of antigen-specific IL-2 and IFN- $\gamma$  in spleen cell cultures from L3-infected mice. This is the first evidence that *Brugia*-reactive Th1 cells are primed upon infection with L3. Therefore, it appears that IL-10 produced during the L3-induced Th2 response *in vivo* may actively suppress the activity of Th1-primed cells. It would be interesting to examine the pattern of cytokines produced by spleen cells from L3-infected IL-10 KO mice, since IL-10 KO mice develop a Th1 response upon infection with *N. brasiliensis* (Kuhn *et al*, 1993). IL-10 has been shown to mediate the down-regulation of parasite antigen-driven proliferation of PBMCs from microfilaraemic humans (King *et al*, 1993) and IFN- $\gamma$  production following infection of BALB/c with *B. malayi* mf or mf extract (Pearlman *et al*, 1993). In addition, depletion of adherent cells from the spleen cell preparation from L3-infected mice and replacement with irradiated spleen cells from uninfected animals results in antigen-stimulated IL-2 and IFN- $\gamma$  production. This would suggest that APC populations, perhaps via IL-10 production, play a role in suppressing antigen-specific Th1 activity in L3-infected mice. Furthermore, the ratio of T cell:APC (3:2) that results in full recovery of mitogen-driven IL-2 and IFN- $\gamma$  production, completely abrogated the secretion of ConA-stimulated Th2 cytokines by T cells from the L3 infected animals. It appears that the established Th2 phenotype of the response can be switched by removal of the resident APC population and replacement with APC from uninfected animals.

The results from these experiments demonstrate that exposure to L3 is a critical early event in the polarization of the immune response, creating an environment in which Th2

responses predominate. IL-4, IL-10 and APC-mediated suppression of mitogen Th1 activity is clearly operative following infection with L3. Furthermore, IL-10 and the APC population, but not IL-4, appears to inhibit antigen-specific Th1 cells during the Th2 polarized response. The suppression of antigen-specific Th1 cells may underlie the Th1 mitogen unresponsiveness in L3-infected mice. The relative contribution of each of these factors to mitogen and antigen Th1 unresponsiveness requires further investigation. In these experiments, it was not possible to determine whether Th1 cells are suppressed from the outset of infection following the preferential expansion of Th2 cells, or whether a Th1/Th0 response occurs initially but switches to a Th2 dominated response before day 12 p.i. Measurement of cytokine expression during the first few days p.i. may distinguish between the different mechanisms which may give rise to the Th2 bias (See Chapter 5).

## CHAPTER 5



## Chapter 5 Infection with L3 induces a Th2-polarised response following activation of an IL-4 producing CD4<sup>+</sup>CD8<sup>-</sup> $\alpha\beta$ T cell

### 5.1. Introduction

The results from the experiments presented in Chapters 3 and 4 demonstrate that a Th2 dominated response can be detected in BALB/c mice at day 12 p.i. with L3, concurrent with suppressed polyclonal and antigen-specific Th1 responses. The experiments in this chapter are focused on examining Th cell polarization during the early period after infection.

It is known that Th1 or Th2 effector cells can differentiate from common IL-2 producing naive CD4<sup>+</sup> precursors and that this process can be influenced by a number of variables. The contribution of cytokines, genetic polymorphisms, the type and dose of antigen, route of administration and costimulation appear to differ depending on the situation, as outlined in the Introduction. However, most studies indicate that the presence of cytokines during T cell priming are the dominant factors driving Th development as demonstrated by *in vitro* and *in vivo* treatment with cytokines and anti-cytokine antibodies and in mice expressing cytokine transgenes or with targeted disruptions in cytokine genes. Of major importance are IL-4, and perhaps IL-10, for Th2 development and IL-12 and IFN- $\gamma$  for Th1 development (as referenced in the Introduction). IL-12, produced rapidly by macrophages and dendritic cells upon exposure to microbial agents and their products, promotes the induction of IFN- $\gamma$  producing Th1 cells. In addition, NK cells may be the predominant source of IFN- $\gamma$  produced early in the immune response (Manetti *et al*, 1993; Scharton and Scott, 1993). Furthermore, when both cytokines are present during primary *in vitro* stimulation, IL-4 dominates over IL-12 for phenotype induction (Hsieh *et al*, 1993; Schmitt *et al*, 1994a), which is thought to reflect the expression of receptors for IL-4, but not IL-12, on naive CD4<sup>+</sup> cells (Lowenthal *et al*, 1988) and the rapid extinction of IL-12 signalling in Th2 cells (Szabo *et al*, 1995).

The pathway of Th2 cell induction is the more complex of the two, since IL-4 is both the stimulus and a product. Therefore, identifying the source of IL-4 at the initiation of primary Th2 responses has been the focus of much recent research in Th cell subset differentiation. Recent results from several systems have identified subpopulations of T cells that are capable of prompt IL-4 production in response to a variety of stimuli. These include unusual T cell populations that express the NK1.1 marker (Zlotnik *et al*, 1992; Yoshimoto and Paul, 1994; Vicari *et al*, 1996). In other systems, the early production of IL-4 has been attributed to "conventional" T cell populations including both CD4<sup>+</sup> T cells (Svetic *et al*, 1993; Gollob and Coffman, 1994; Reiner *et al*, 1994; Schmitz *et al*, 1994; Demeure *et al*, 1995) and  $\gamma\delta$  T lymphocytes (Ferrick *et al*, 1995), although the expression of NK1.1 on these populations was not examined. Furthermore, there are potential non-T cell sources of preformed IL-4 that could act at the initiation of immune responses, including mast cells and basophils (Plaut *et al*, 1989; Seder *et al*, 1991; Bradding *et al*, 1992; Williams *et al*, 1993). In a recent study an early burst of IL-4 transcription, observed following infection with *Schistosoma* eggs, was thought to be produced by eosinophils (Sabin and Pearce, 1996).

Naive T cells from BALB/c mice appear to have an intrinsic capacity to develop towards the Th2 phenotype (Hsieh *et al*, 1995). However, in BALB/c mice infected with *L. major* promastigotes, that fail to provide a strong stimulus for driving Th phenotype development, CD4<sup>+</sup> cells from the draining LN contained transcripts for IL-2, IL-4 and IFN- $\gamma$  at day 4 p.i., indicative of the induction of a Th0 or mixed Th population early after infection. The response eventually becomes Th2-dominated by day 14 p.i., by which time IFN- $\gamma$  mRNA levels had declined while IL-4 mRNA expression remained high (Reiner *et al*, 1994). In contrast, infection of BALB/c mice with *H. polygyrus* induces early cytokine gene expression that is restricted to Th2-associated cytokines (Svetic *et al*, 1993), consistent with the propensity of nematode parasites to induce polarized Th2 responses. After only 12hrs p.i. IL-5, IL-9 and IL-3 mRNA levels were elevated in the Peyer's patches. IL-4 mRNA levels became elevated

by 4 to 6 days after infection, but neither IL-2 or IFN- $\gamma$  mRNA levels were upregulated at any time during the primary response. Using athymic mice and anti-CD4 or anti-CD8-MAb treated BALB/c mice, the early increases in IL-5, IL-9 and IL-3 gene expression was shown to be T-cell-independent, but were T-cell-dependent by day 4-6 p.i. IL-4 gene expression was restricted to the CD4<sup>+</sup> population. Therefore, the response to infection with *H. polygyrus* appears to be skewed in the Th2 direction from the outset.

In the studies described in this chapter, it was decided to use semi-quantitative RT-PCR to determine whether a specific pattern of Th cytokine gene expression could be detected at the early stages after primary infection of BALB/c mice with L3. The cellular source of individual cytokines could be assessed by magnetic cell selection before RNA preparation. For comparative purposes, the early cytokine transcription pattern elicited by mf, that induce a primary Th1 response (Chapter 3), was analyzed in parallel. The results of these experiments should discriminate between the following mechanisms:

- (a) Th2 cells are preferentially activated from the outset,
- (b) The eventual Th2 response develops from an early Th1, mixed or Th0 response,
- (c) The eventual Th2 response arises by default due to Th1 cell anergy, that has been postulated to underly the impaired antigen-specific Th1 responses in human filarial infection.

## 5.2. Results

### 5.2.1. Verification of the RT-PCR method for quantitation of IL-2, IFN- $\gamma$ , IL-4, IL-10 and $\beta$ -actin mRNA expression in LN cells

In preliminary experiments, the appropriate cycle number was determined for each gene at which a linear relationship between the amount of input RNA and final PCR product could be demonstrated, as described previously by Svetic *et al* (1991). Briefly, total RNA obtained from popLN cells of infected mice (known to contain high amounts of specific cytokine mRNA as determined from initial experiments) was serially diluted 1/1.25 ten times. RT-PCR was then performed on each dilution over a range of amplification cycles for IL-4, IL-10, IL-2, IFN- $\gamma$  and  $\beta$ -actin. After electrophoresis, Southern blotting and hybridisation with the gene specific probe the resultant autoradiograph was analyzed by densitometry. Titration curves were then drawn by expressing the signals detected relative to the amount of template RNA. Such dilution series demonstrated that, at the optimal cycle number for each gene (IL-2[20], IFN- $\gamma$ [25], IL-4[25], IL-10[20] and  $\beta$ -actin[15]), the amount of PCR product is linearly proportional to the amount of starting RNA and that 1.25-fold differences in RNA levels could be detected. Figure 5.1 shows a representative autoradiograph (a) and the densitometric analysis (b) of the IL4 RT-PCR signal at the optimal 25 cycles as a function of the amount of input RNA. Figure 5.2 shows that when the cycle number was increased to 35, the IL-4 RT-PCR signal did not vary relative to the 1.25-fold change in the amount of input RNA, indicating that the individual PCR reactions had reached saturation level and, therefore, at 35 cycles any differences in IL-4 expression would not be quantitative.

Using the optimal cycle number determined for each gene, this method was then used to detect quantitative differences in relative amounts of IL-4, IL-10, IL-2 and IFN- $\gamma$  mRNA between experimental samples. To verify that equal amounts of RNA were added to each RT-PCR reaction within an experiment, the constitutively expressed gene

**FIGURE 5.1. The autoradiographic (a) and the densitometric analysis (b) of IL-4 RT-PCR signal at 25 cycles as a function of the amount of input RNA.**

Total RNA obtained from popLN cells of infected mice was serially diluted 1/1.25 ten times. RT-PCR for 25 cycles was performed on each dilution, as described in the Materials and Methods (2.3), and after electrophoresis, Southern blotting and hybridisation the resultant autoradiograph was analyzed by densitometry. Each density value was normalised to the value obtained with the maximum amount of RNA (2.5µg=100%). The straight line drawn represents a 1/1.25 change in the RT-PCR signal over the dilution series. The best fit curve of the actual dilution series was not significantly different from this curve as determined by linear regression analysis.

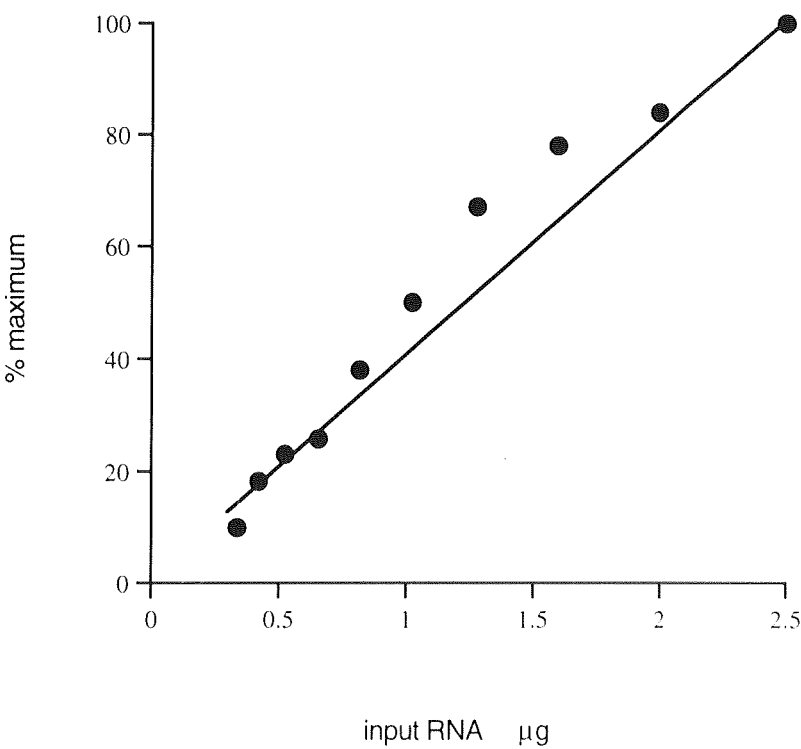
**Figure 5.1**

IL-4 signal at 25 cycles

**(a)**



**(b)**



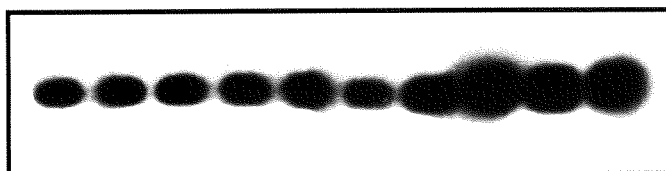
**FIGURE 5.2. The autoradiographic (a) and the densitometric analysis (b) of IL-4 RT-PCR signal at 35 cycles as a function of the amount of input RNA.**

As for Fig. 5.1 except that RT-PCR was performed on each dilution for 35 cycles. The straight line drawn represents a 1/1.25 change in the RT-PCR signal over the dilution series. The best fit curve of the actual dilution series was significantly different from this curve as determined by linear regression analysis.

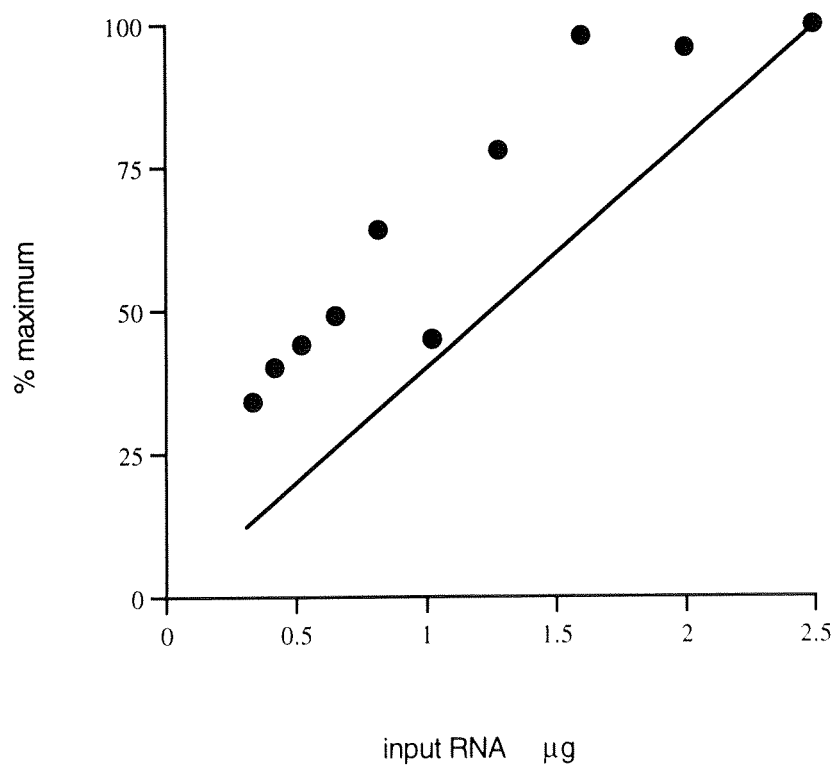
**Figure 5.2**

IL-4 signal at 35 cycles

**(a)**



**(b)**





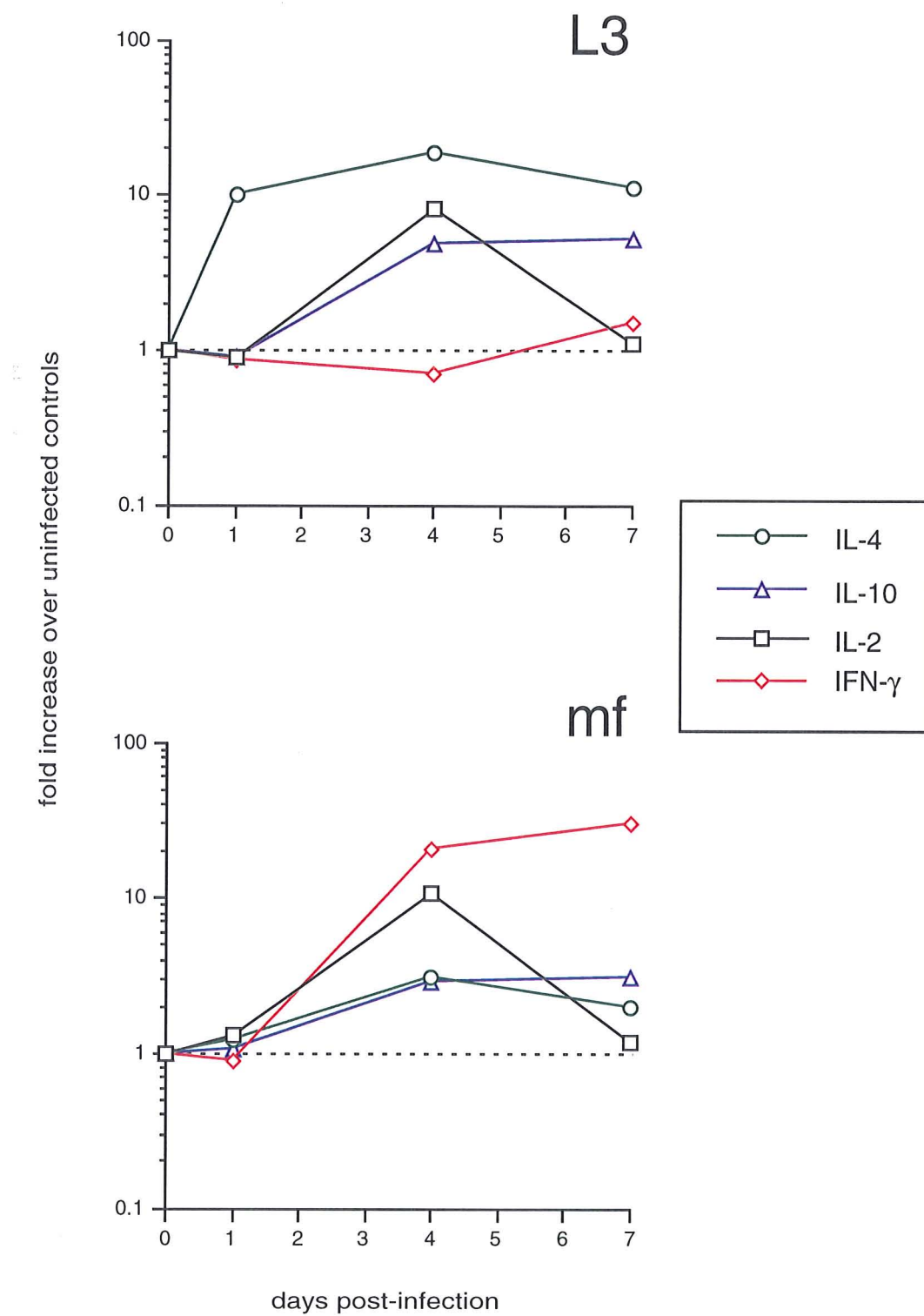
$\beta$ -actin was reverse transcribed and amplified for each sample. All values in all experiments were individually normalised to  $\beta$ -actin.  $\beta$ -actin did not show changes greater than two times between compared groups in any of the experiments shown. Furthermore, several autoradiographic exposures of each Southern blot were analysed to ensure that the signal from each band was in the linear range of the film response. Although this method does not allow for the determination of absolute levels of gene expression or comparison between levels of different cytokine mRNA, it does permit measurement of changes in the relative abundance of particular cytokines between different samples.

### **5.2.2. L3 and mf induce a contrasting pattern of cytokine gene expression early after infection**

BALB/c mice were infected in the hind footpads with L3 or mf and killed 24h, 4d or 7d later. Control animals received an equivalent volume of HBSS. Analysis of cytokine mRNA levels in the draining popLN by semi-quantitative RT-PCR revealed a highly stage-specific and polarized response from the outset of infection (Fig. 5.3). The most striking observation was the early burst in IL-4 transcription within 24h p.i. with L3. It was the only cytokine transcript detectable at this time point and was not induced following infection with mf. In the representative experiment shown in Figure 5.3, there was a 10-fold increase in IL-4 transcription in L3-infected mice over uninfected controls. The increase in abundance of IL-4 transcripts varied from experiment to experiment, but was always in the range of 10-20-fold. By day 4 and 7 p.i. with L3, IL-4 and IL-10 mRNA levels were elevated, in the absence of any IFN- $\gamma$  induction. This contrasts with the situation following infection with mf where elevated levels of IFN- $\gamma$ , but not IL-4 or IL-10, were detected at day 4. By day 7 p.i. with mf the cytokine profile showed a low level of IL-4 and IL-10 transcription (2-3-fold more than uninfected controls), but was dominated by high levels of IFN- $\gamma$  mRNA production (30-fold more than uninfected controls). However, both mf and L3 induced identical kinetics of IL-2 mRNA induction, peaking at day 4 and rapidly declining to baseline levels by day 7 p.i.

**FIGURE 5.3. Cytokine mRNA expression in the popLN at early time points after infection with *B. pahangi* L3 or mf**

Balb/c mice were injected with 30 L3 (top),  $6 \times 10^4$  mf (bottom) or HBSS and popLN harvested at the indicated time points for RNA extraction. RT-PCR, electrophoresis, Southern blotting, hybridisation and autoradiography were performed as described in Materials and Methods (2.3). mRNA levels for each designated cytokine were expressed relative to the level in the popLN of uninfected animals, that were assigned a value of one. cDNA concentrations were standardised in individual samples by normalising to the constitutive gene  $\beta$ -actin, that did not show changes greater than 2-fold between compared samples. The results represent one of two comparable time course experiments.



### **5.2.3. The L3 induced IL-4 burst at 24h is produced by a CD4<sup>+</sup>CD8<sup>-</sup> $\alpha\beta$ T cell population**

In an attempt to define the cellular source of the early IL-4, popLN cells from mice 24h p.i. with L3 were separated into defined cell populations using specific MAbs coupled to magnetic beads. In separate experiments MAbs to CD4, CD8, CD3 and Thy1.2 were used to perform the purification. The purity of the designated selections exceeded 85-95% as assessed by FACS analysis. RNA was extracted from the positive and negative fractions and from the unseparated popLN population, reverse transcribed and the resultant cDNA was analyzed for IL-4 transcripts and standardised to  $\beta$ -actin. Fig. 5.4 shows a compilation of representative autoradiographs. Using this approach the burst of IL-4 at 24h can be clearly defined within the CD4<sup>+</sup> CD8<sup>-</sup> population. To determine whether the IL-4 producers within the DN population were T or non-T cells, a MAb to CD3 was used to select the whole 24h popLN into CD3<sup>+</sup> and CD3<sup>-</sup> fractions. While IL-4 transcripts were detectable in the unseparated popLN cells from L3-infected mice, no transcripts could be detected in the CD3<sup>-</sup> population, indicating that the burst of IL-4 is unlikely to derive from a non-T cell source. However, in this and two additional experiments, no mRNA could be recovered from the positively selected population. This could be due to rapid mRNA turnover following mitogenic signalling via CD3, despite the fact that all incubations were carried out at 4°C.

Two approaches were then used to confirm directly that the early IL-4 is T cell derived. In the first, Thy1.2 was chosen as an alternative T cell marker. Fig. 5.4 shows that following selection using anti-Thy1.2 Mab, the IL-4 signal at 24h p.i. was present in the Thy1.2<sup>+</sup> population. No difficulties in RNA recovery were found using MAb to the Thy1.2 antigen for magnetic cell selection. In the second approach, T cells from popLN cells pooled from mice 24h p.i. with L3, were positively selected using murine T cell enrichment mini-columns from R&D Systems (See 2.3.5). RT-PCR was then performed on RNA prepared from the T cell purified population (95.5% CD3<sup>+</sup> by FACS) and unseparated LN cells from both L3-infected and uninfected mice. Fig. 5.5 shows that

**FIGURE 5.4. Cellular source of the IL-4 burst at 24h p.i. with *B. pahangi* L3 by magnetic cell selection.**

BALB/c mice were injected with 30 L3 or HBSS and popLN harvested after 24h. In separate experiments popLN from infected animals were selected into positive (+) and negative (-) populations using MAbs to CD4, CD8, CD3 and Thy1.2 coupled to magnetic beads. The purity of the depleted populations was > 90% as assessed by FACS, CD4 (91.1%), CD8 (97.0%), CD3 (95.2%), Thy1.2 (95.8%). Total RNA, isolated from the separated populations (+ and -) and unseparated popLN cells from infected (US) and uninfected animals (Con), was analyzed for the expression of IL-4 and  $\beta$ -actin by RT-PCR, as described in Materials and Methods (2.3). A compilation of representative autoradiographs is shown opposite. No RNA could be recovered from the CD3<sup>+</sup> population (See Results). Comparable results were obtained in independent experiments.

**FIGURE 5.5. Confirmation of a T cell source for the IL-4 burst at 24h p.i. with *B. pahangi* L3**

BALB/c mice were injected with 30 L3 or HBSS and popLN harvested after 24h. T cells were purified from popLN from infected animals using T cell enrichment columns (R&D systems), as described in Materials and Methods. The T cell enriched population was 95.5% CD3<sup>+</sup> as assessed by FACS. Total RNA, isolated from the T cell enriched population (T) and unseparated popLN cells from infected (US) and uninfected animals (Con), was analyzed for the expression of IL-4 and  $\beta$ -actin by RT-PCR. Representative autoradiographs are shown above.

Figure 5.4

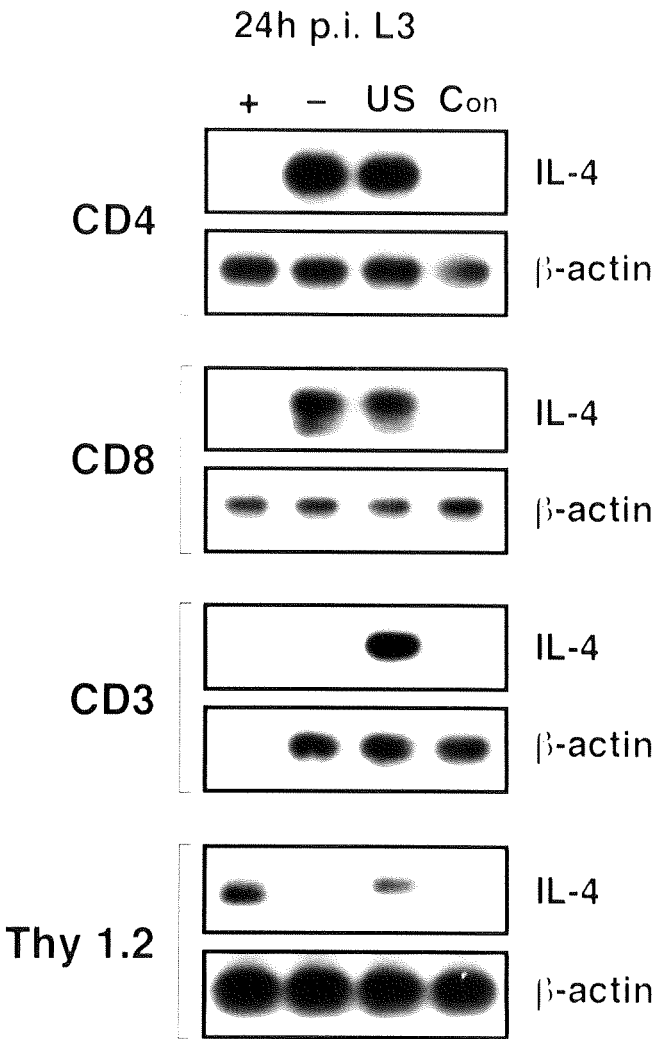
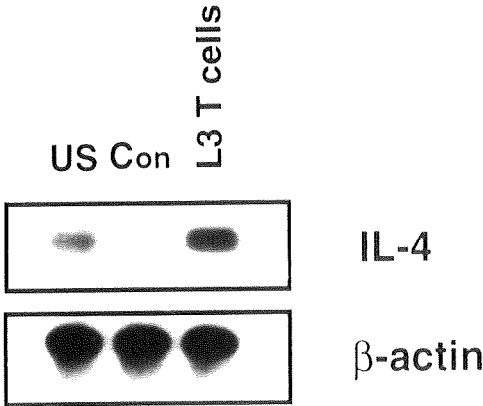


Figure 5.5



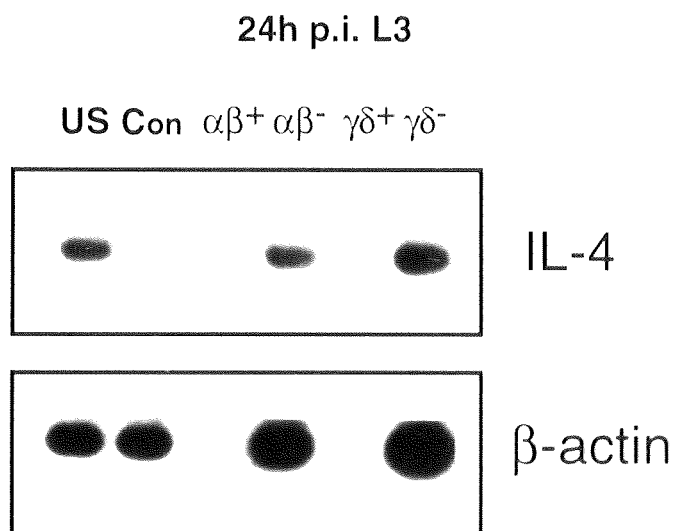
**FIGURE 5.6. IL-4 expression at 24h p.i. in the  $\gamma\delta$  T cell depleted population**

BALB/c mice were injected with 30 L3 or HBSS and popLN harvested after 24h. PopLN from infected animals were selected into positive (+) and negative (-) populations using MAbs to  $\gamma\delta$  and  $\alpha\beta$  TCR coupled to magnetic beads. The purity of the  $\gamma\delta^-$  population was 99.8%. The purity of the  $\alpha\beta^-$  population was 77.0%. Total RNA, isolated from the separated populations (+ and -) and unseparated popLN cells from infected (US) and uninfected animals (Con), was analyzed for the expression of IL-4 and  $\beta$ -actin by RT-PCR. Representative autoradiographs are shown above. No RNA could be recovered from the  $\gamma\delta^+$  or  $\alpha\beta^+$  populations (see text for more detail).

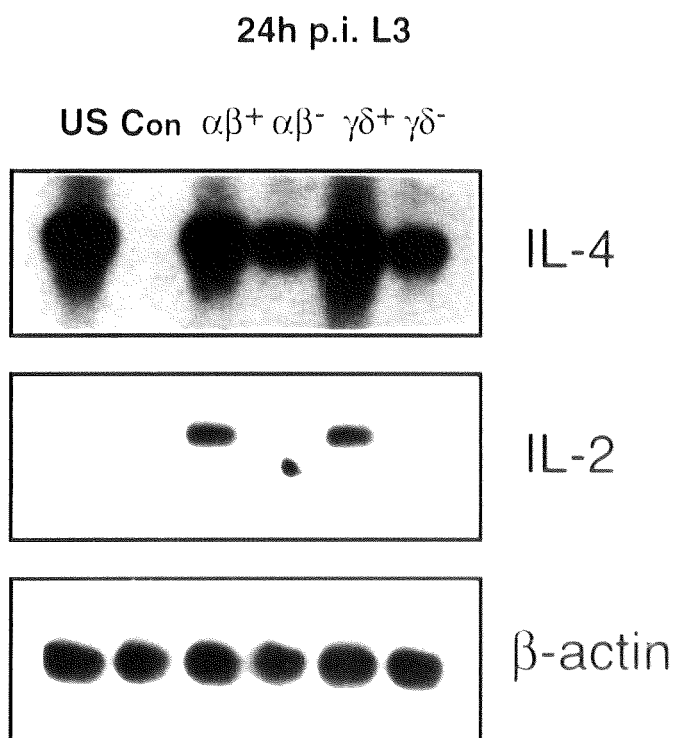
**FIGURE 5.7. Confirmation of IL-4 expression at 24h p.i. in the  $\gamma\delta$  T cell depleted population**

Mice were infected and cell selections were performed, as described in the legend to Fig. 5.6. In this experiment RNA was recovered from the  $\gamma\delta^+$  or  $\alpha\beta^+$  populations but no determination of the purity of the depleted populations by FACS analysis was performed. Total RNA, isolated from the separated populations (+ and -) and unseparated popLN cells from infected (US) and uninfected animals (Con), was analyzed for the expression of IL-4, IL-2 and  $\beta$ -actin by RT-PCR. Representative autoradiographs are shown above.

**Figure 5.6**



**Figure 5.7**



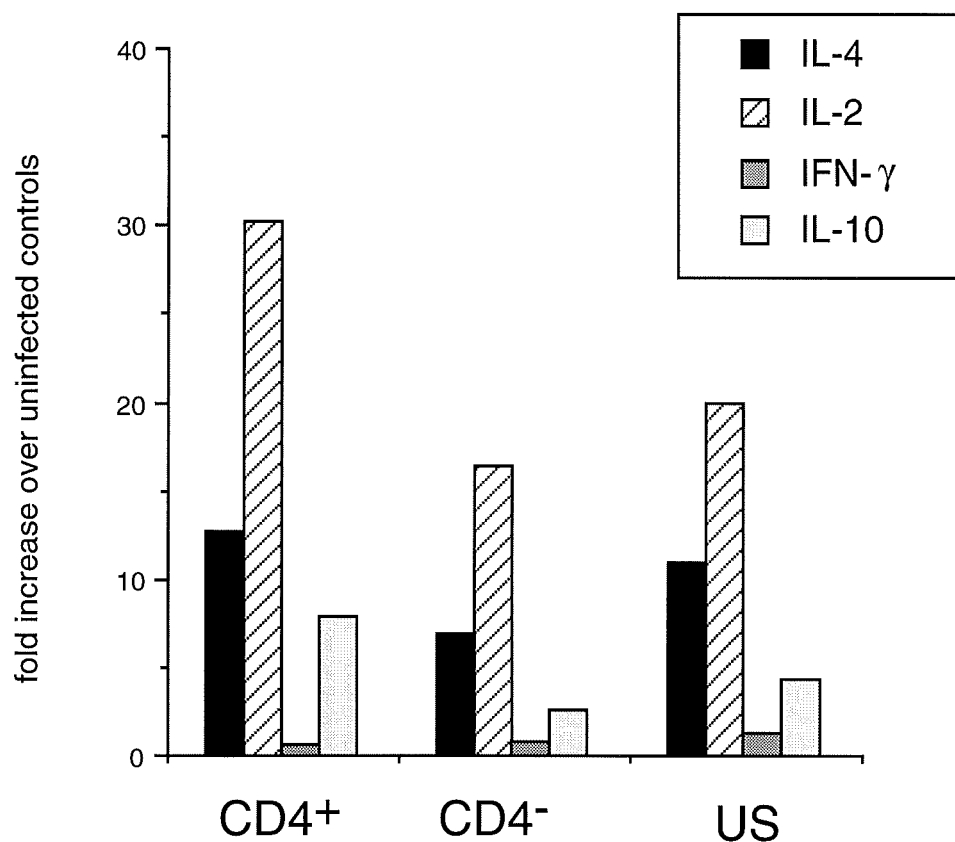


IL-4 mRNA expression is up-regulated in the enriched T cell population of LN cells from L3-infected mice.

This analysis clearly demonstrated that at 24h p.i. with L3, the IL-4 transcripts derived from a CD4<sup>+</sup> CD8<sup>-</sup> T cell. It was of interest to further investigate whether the source of the IL-4 was an  $\alpha\beta$  or a  $\gamma\delta$  T cell, as recent reports suggest that  $\gamma\delta$  T cells can be a source of early IL-4 (Ferrick 1995, Vicari 1996). Pop LN cells at 24 h p.i. were separated using antibodies to the  $\gamma\delta$  and  $\alpha\beta$  TCR. However, RNA could not be recovered consistently from positively selected  $\gamma\delta$  and  $\alpha\beta$  cells. Despite this problem, the results of these experiments suggest that  $\alpha\beta$  T cells rather than  $\gamma\delta$  T cells are the likely producers of the early burst of IL-4. Fig. 5.6 shows that IL-4 transcripts were present in the  $\gamma\delta^-$  population.  $\gamma\delta$  T cells were not detected in the depleted population as assessed by FACS (US=2.8%  $\gamma\delta^+$ ,  $\gamma\delta$ -depleted=0.2%  $\gamma\delta^+$ ). However,  $\alpha\beta$  selection was not as successful. Despite a decrease in total cell numbers following selection (US total cell number= $2.5 \times 10^7$ ,  $\alpha\beta$ -depleted total cell number= $1.1 \times 10^7$  i.e. 44% recovery),  $\alpha\beta$  T cells did not appear to be depleted as assessed by FACS staining (US=68.2%  $\alpha\beta^+$ ,  $\alpha\beta$ -depleted=77.0%  $\alpha\beta^+$ ). Therefore, it cannot be concluded that IL-4 transcripts detected in the " $\alpha\beta^-$  population" are derived from  $\alpha\beta^-$  T cells. Furthermore, when RNA was successfully isolated and used for RT-PCR, both IL-4 and IL-2 transcripts were detected in the  $\alpha\beta^+$  and  $\gamma\delta^+$  populations (Figure 5.7). However, since IL-2 mRNA is not expressed in unseparated LN population at 24 h p.i., it appears that the magnetic selection protocol used in this study activates positively selected  $\gamma\delta$  and  $\alpha\beta$  cells. Therefore, it was not possible to directly assess the contribution of  $\alpha\beta$  and  $\gamma\delta$  cells to the early burst of IL-4.

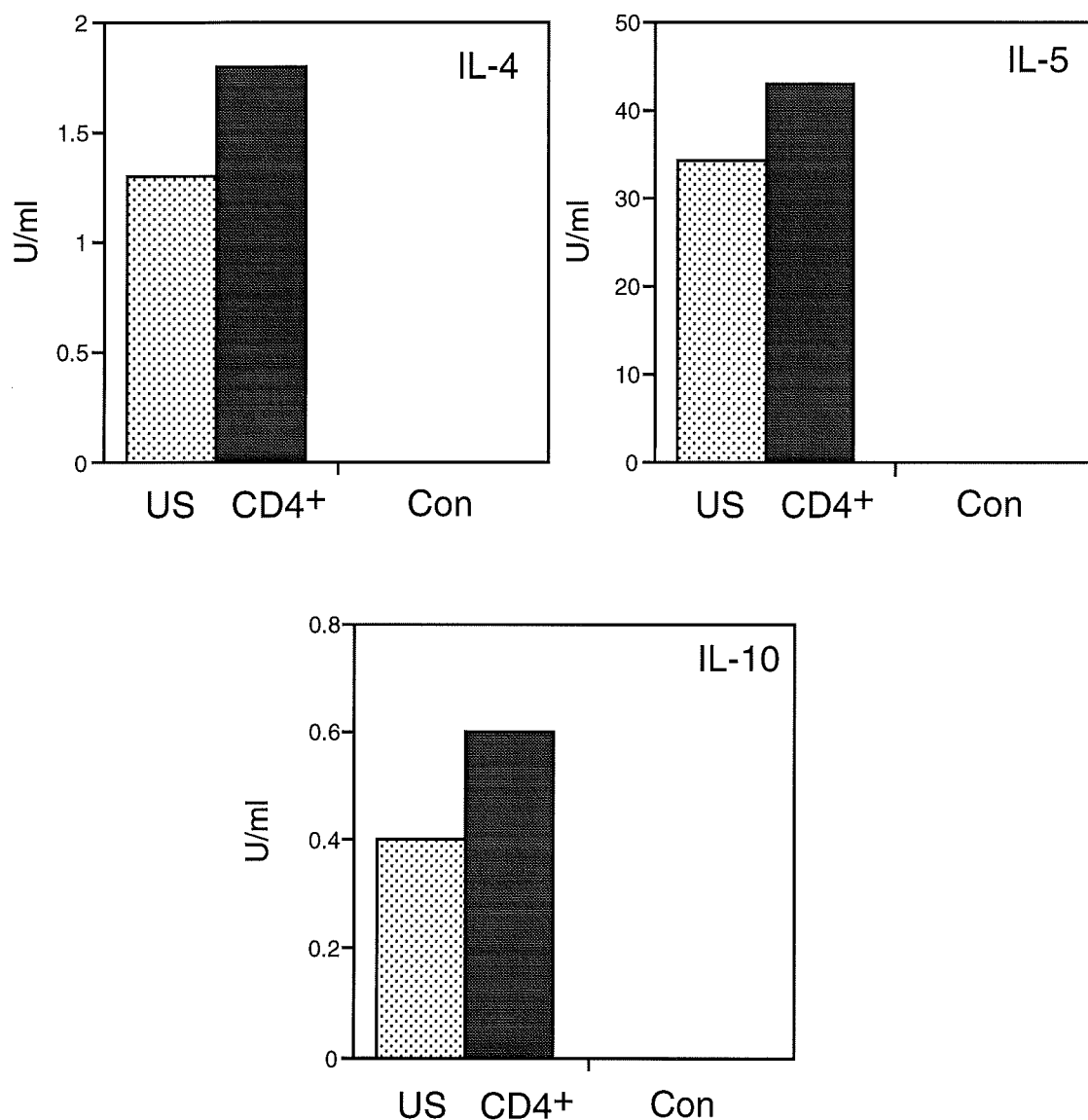
#### **5.2.4. At day 4 p.i. with L3 popLN CD4<sup>+</sup> cells express IL-2, IL-4 and IL-10 mRNA**

In order to determine whether the burst of IL-4 transcription at 24h p.i. with L3 results in a Th2 response in the draining lymph node, a CD4 selection was performed on



**FIGURE 5.8.** Cytokine gene expression by CD4<sup>+</sup> and CD4<sup>-</sup> cells at day 4 p.i. with *B. pahangi* L3

Balb/c mice were injected with 30 L3 or HBSS and popLN harvested after 4d. Cells were separated into CD4<sup>+</sup> and CD4<sup>-</sup> populations. Cytokine mRNA analysis was performed on unseparated (US) or separated populations and band intensities were quantified and expressed as described in legend to Fig. 5.3.



**FIGURE 5.9. Antigen-specific cytokine secretion *in vitro* at day 10 p.i. with *B. pahangi* L3**

BALB/c mice were injected with 30 L3 or HBSS and popLN harvested after 10d. CD4<sup>+</sup> cells were separated on a Minimacs column and purified CD4<sup>+</sup> cells were plated out at  $4 \times 10^6$ /ml in the presence of  $6 \times 10^6$  irradiated syngeneic spleen cells/ml. Unseparated popLN cells (US) from L3 infected mice and uninfected controls (Con) were incubated at  $1 \times 10^7$ /ml. Cells were stimulated with *B. pahangi* antigen (10 $\mu$ g/ml) and cytokines assayed by 2-site ELISA.

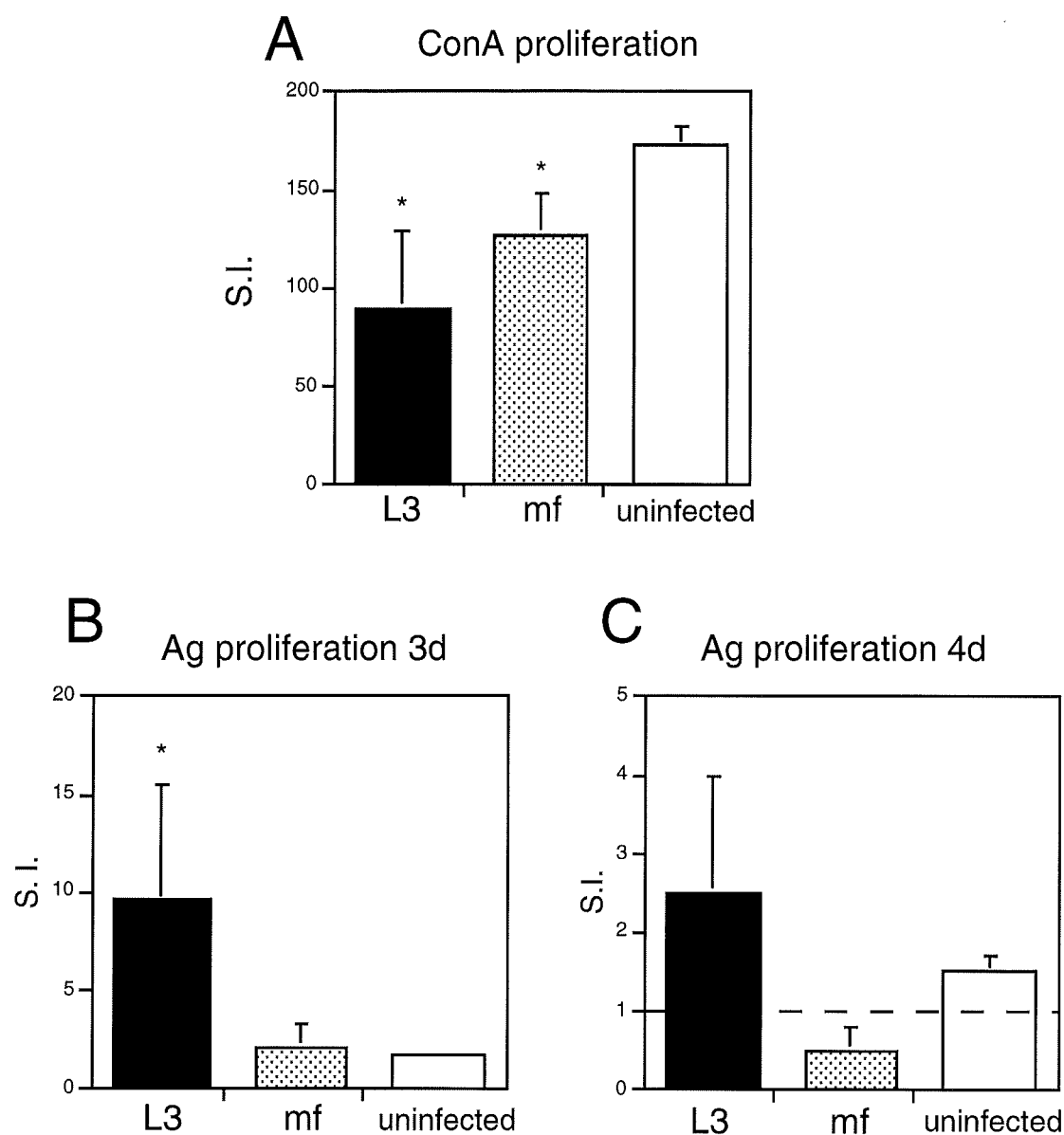
popLN cells at day 4 p.i. The results of this experiment (Fig. 5.8) show that CD4<sup>+</sup> cells clearly contribute to the IL-4 signal and also to the elevated levels of IL-2 and IL-10 mRNA day 4 p.i.. Expression of IL-4 and IL-2 mRNA could also be detected in the CD4<sup>-</sup> population. However, no IFN- $\gamma$  signal is detectable in either the CD4<sup>+</sup> or CD4<sup>-</sup> populations at day 4 p.i..

#### **5.2.5. By day 10 p.i. with L3 popLN CD4<sup>+</sup> cells secrete Th2 cytokines exclusively**

The results described above indicate that L3 induce a dominant Th2 response, at the mRNA level, from the outset of infection. However, it was important to confirm whether Th2 cytokines were actually secreted in response to infection with the L3 by this route. To achieve this, popLN were removed at day 10 p.i., by which time an antigen-specific Th cell response can be detected *in vitro* upon re-stimulation of lymphocytes. CD4<sup>+</sup> cells were purified using Minimacs columns and the profile of cytokine secretion monitored in response to stimulation with parasite antigen. Fig. 5.9 shows that popLN cells from L3 infected mice produce exclusively Th2 cytokines and that CD4<sup>+</sup> cells were the source of IL-4, IL-5 and IL-10. No antigen-specific IL-2 or IFN- $\gamma$  was detected (data not shown). Therefore, measurement of cytokine protein secretion at day 10 p.i. correlates well with the analysis of cytokine mRNA production.

#### **5.2.6. The immune response of C57BL/6 mice at day 12 p.i. with L3 or mf**

Since the possible source of the early IL-4 may be NK1<sup>+</sup> T cells, preliminary experiments were performed to examine the response of the NK1.1<sup>+</sup> mouse strain, C57BL/6, at day 12 following s.c. infection (in "the scruff of the neck") with L3 and mf. Figure 5.10 and Tables 5.1 and 5.2 show that C57BL/6 mice produce an almost identical profile of proliferation, cytokine and antibody production to that of BALB/c mice in response to infection with L3 and mf. Spleen cells from L3-infected C57BL/6 mice exhibit a significantly reduced proliferative response to ConA compared to uninfected controls (52% reduction,  $p = 0.004$ ), yet proliferate vigorously in response to



**FIGURE 5.10. Proliferative responses of spleen cells from three groups of C57BL/6 mice to ConA and *B. pahangi* adult Ag.**

C57BL/6 mice were injected s.c. with 50 L3,  $1 \times 10^4$  mf or HBSS (uninfected control). At day 12 p.i. proliferation of spleen cells at  $5 \times 10^6$  cells/ml to (A)  $1 \mu\text{g/ml}$  of ConA or (B)  $10 \mu\text{g/ml}$  of *B. pahangi* adult Ag was measured by uptake of  $^3\text{H}$  thymidine after 48h (ConA) or 72h (Ag) of culture. The means of triplicate wells are expressed as stimulation indices (S.I.) = cpm with ConA or Ag/cpm with medium alone. The S.I.s represent the mean  $\pm$  SD of 5 animals per group. \* Significant difference ( $p < 0.05$ ) compared to mice infected with mf or uninfected controls.

**TABLE 5.1. Cytokine responses of spleen cells from three groups of C57BL/6 mice<sup>a</sup> to ConA and *B. pahangi* adult antigen**

stimulus	cytokine levels (U/ml) <sup>b</sup>			
	L 3	Mf	uninfected	
Ag	0	0	0	<b>IL-2</b>
ConA	64.5 ± 34.1	147.8 ± 83.5	108.4 ± 86.9	
Ag	0	20.0 ± 8.4 *	0	<b>IFN-γ</b>
ConA	50.4 ± 22.3 *	153.6 ± 31.3	303.8 ± 225.1	
Ag	0	0	0	<b>IL-4</b>
ConA	0.62 ± 0.57 *	0	0	
Ag	15.2 ± 1.4 *	0	0	<b>IL-5</b>
ConA	15.0 ± 5.9 *	0.4 ± 0.3	1.0 ± 1.1	
Ag	0.7 ± 0.2 *	0	0	<b>IL-10</b>
ConA	1.2 ± 0.3 *	0	0	

<sup>a</sup> Mice were injected s.c. with 50 L3, 1x10<sup>5</sup> mf or HBSS (uninfected).

<sup>b</sup> At day 12 p.i. cytokine levels in 48h supernatants from spleen cells at 1x10<sup>7</sup> cells/ml in response to ConA (5μg/ml) or *B. pahangi* adult Ag (10μg/ml) were measured by 2-site ELISA with reference to standards of known concentration (U/ml). A value of 0 indicates a reading below the sensitivity of the assay. The values are means ± SD of 5 animals per group. \* Significant difference ( $p < 0.05$ ) between infected mice and uninfected controls. Assay sensitivities: IL-2 ConA=1.953U/ml, Ag=3.906U/ml; IFN-γ ConA=1.953U/ml, Ag 7.812U/ml; IL-4 ConA=0.391U/ml, Ag=0.781U/ml; IL-5 ConA=0.195U/ml, Ag=1.563U/ml; IL-10 ConA=0.391U/ml, Ag=0.391U/ml.

**TABLE 5.2. Parasite-specific serum antibody production in C57BL/6 mice infected with L3**

	IgG	IgG1	IgG2a	IgE
L3	0.619 ± 0.118*	0.618 ± 0.364*	0.121 ± 0.006	0.434 ± 0.120*
uninfected	0.137 ± 0.016	0.126 ± 0.017	0.120 ± 0.010	0.291 ± 0.032

Animals were injected s.c. with 50 L3 or HBSS (uninfected). Antigen-specific antibody levels for IgG, IgG1, IgG2a and IgE, in serum collected at day 12 p.i., were measured by ELISA using *B. pahangi* adult extract as the target antigen. The values represent the means ± S.D. of 5 animals per group. \* Significant difference ( $p < 0.05$ ) compared to uninfected controls.

antigen (S.I.=9.9,  $p = 0.004$  compared to uninfected controls) (Fig. 5.10A). Furthermore, spleen cells from L3-infected C57BL/6 mice secreted elevated levels of antigen-stimulated IL-5 and IL-10, but not IL-2 and IFN- $\gamma$ , while stimulation with ConA resulted in increased levels of IL-4 and reduced levels of IFN- $\gamma$  ( $p = 0.004$ , compared to the other two groups) (Table 5.1). L3-infected C57BL/6 mice also produced elevated levels of serum parasite-specific IgG, IgG1 and IgE (all values  $p=0.004$  compared to uninfected controls), but not IgG2a (Table 5.2). As further evidence that these mice respond in a similar manner to BALB/c mice, spleen cells from C57BL/6 mice that had been infected with  $1 \times 10^5$  mf exclusively produce antigen-specific IFN- $\gamma$  (Table 5.1), but were unable to respond to antigen at after 3 days in culture and exhibited mean S. I.<1 after 4 days (Fig. 5.10B). However, in this experiment, spleen cells from mf-infected C57BL/6 mice also exhibited a reduced capacity to proliferate in response to ConA (26% reduction compared to uninfected controls,  $p = 0.008$ ). While the level of reduction was less than that of spleen cells from BALB/c mice infected with L3 (64-98%), an impaired ConA proliferative response was sometimes observed following infection of C57BL/6 and BALB/c mice with this higher dose of mf ( $1 \times 10^5$ ) and may reflect an underlying wave of apoptosis in the spleens of these mice (See Chapter 6).

#### **5.2.7. I.V. injection with L3 induces a burst of IL-4 mRNA expression within hours in the spleens of BALB/c and C57BL/6 mice.**

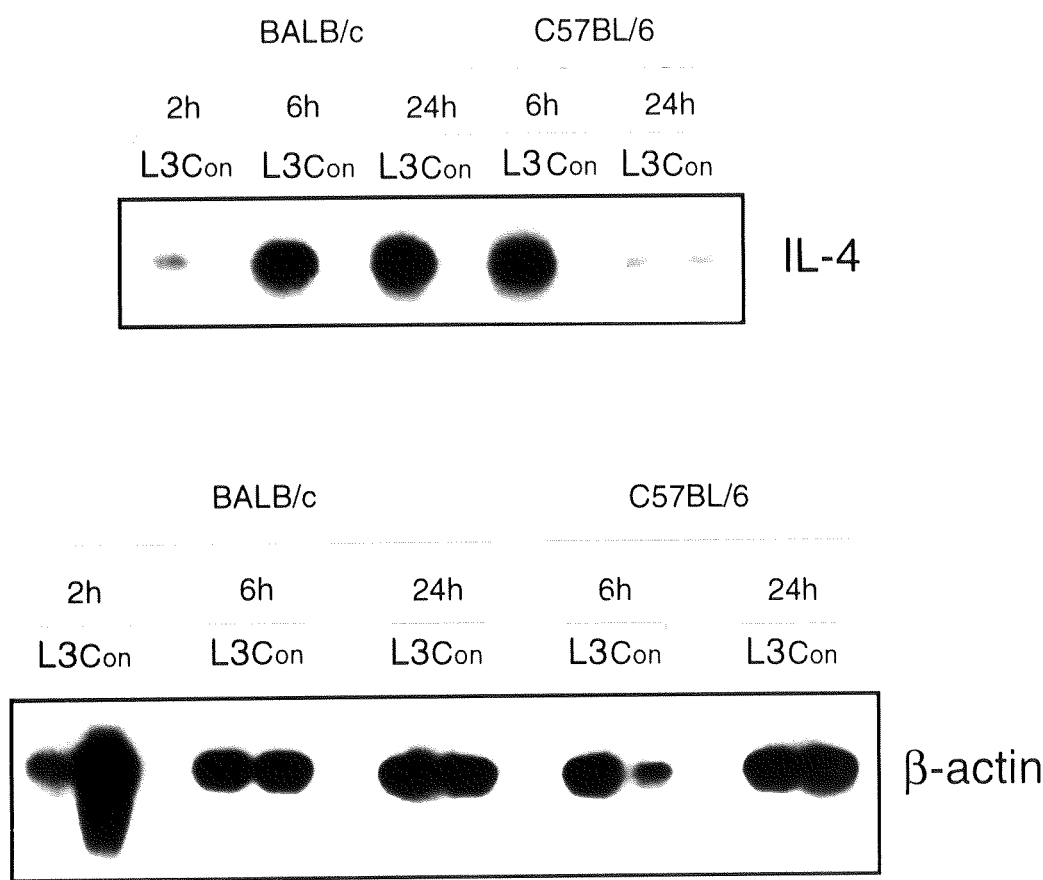
In this experiment BALB/c and C57BL/6 mice were injected i.v. into the tail vein with 50 L3 or HBSS and the spleens removed for RNA extraction at 2h, 6h and 24h. RT-PCR was performed on diluted cDNA (1/20) for IL-4 and  $\beta$ -actin. Figure 5.11 shows that IL-4 mRNA can be detected in BALB/c spleens at only 2h p.i. with L3. The level of IL-4 transcription then increases enormously by 6h p.i. and remains at that level until at least 24h p.i. The kinetics of IL-4 mRNA induction appears to be different in C57BL/6 spleen following i.v. injection of L3. A massive burst of IL-4 was detected at 6h p.i. that was absent by 24h. It was not possible to measure IL-4 expression in



**FIGURE 5.11. IL-4 mRNA expression in the spleens of BALB/c and C57BL/6 mice following i.v. injection of L3.**

Groups of BALB/c and C57BL/6 mice were injected i.v. into the tail vein with 50 L3 or HBSS and the spleens removed for RNA extraction at 2h, 6h and 24h. RT-PCR was performed on diluted cDNA (1/20) for IL-4 and  $\beta$ -actin and representative autoradiographs are shown above.

Figure 5.11



C57BL/6 spleen at the earliest time point since, in two separate experiments, it was not possible to amplify from the RNA extracted.

### 5.3. Discussion

In this chapter, semi-quantitative RT-PCR was employed to analyze cytokine induction during the first few days of infection with L3, known to be a potent Th2 inducer in naive BALB/c mice (as described in Chapters 3 and 4). Measurement of cytokine mRNA levels in defined cell populations revealed that the L3 stimulates a burst of IL-4 gene transcription from a population of DN (CD4<sup>-</sup>CD8<sup>-</sup>) T cells, within 24 hours of infection. The cell population producing IL-4 was defined as a DN T cell by magnetic cell selections using MAbs to CD4, CD8, CD3 and Thy1.2 and by using T cell enrichment columns. The early burst of IL-4 gives rise to a Th2 response in the draining popLN, with both IL-4 and IL-10 mRNA levels elevated at day 4 and day 7 p.i., in the absence of any IFN- $\gamma$  induction. As would be predicted, by day 4 p.i. CD4<sup>+</sup> cells contribute to the increase in IL-4 and IL-10 transcription. Consistent with the gene expression data, CD4<sup>+</sup> popLN cells from L3 infected animals at day 10 p.i. secrete IL-4 and IL-10, but no IL-2 or IFN- $\gamma$ , when re-stimulated *in vitro* with parasite antigen.

While the focus of the experiments in this chapter was on the early response to L3, mice were also infected with mf since injection of live mf has been shown to induce a primary Th1 response at day 12/14 p.i. (Chapter 3, Lawrence *et al*, 1994). In contrast to the results obtained with the L3, the pattern of cytokine gene expression following infection with mf was dominated at day 7 p.i. by high levels of IFN- $\gamma$  mRNA, in the absence of any IL-4 or IL-10 transcription. IFN- $\gamma$  was not upregulated at 24 hrs p.i. with mf (although it may have been expressed earlier than, or shortly after, 24h). In view of the critical role of IL-12, but not IFN- $\gamma$ , in selecting for Th1 differentiation, it would be interesting to determine whether mf induce an early burst of IL-12. However, low levels of IL-4 and IL-10 mRNA transcription were detected at day 4 p.i. with mf in the presence of IL-2 and IFN- $\gamma$  expression, indicative of an early Th0 or mixed Th population. This would suggest that IL-12 transcription, if induced, may be delayed, as is the case following infection with *L. major* promastigotes, even in resistant mouse strains that do eventually make Th1 dominated responses. Alternatively, extracellular

pathogens, like *Brugia* mf, may employ different mechanisms to induce a Th1 response to those used by intracellular pathogens like *Listeria* that rely on the rapid induction of IL-12 (Hsieh *et al*, 1993). Since the burst of IL-4 at 24h p.i. was undetectable in mf-infected mice, L3-induced early IL-4 production by a DN T cell population may be critically important for stage-specific Th2 development.

The DN (CD4<sup>-</sup> CD8<sup>-</sup>) IL-4 producing T cells identified in this study are likely to be  $\alpha\beta$ <sup>+</sup> T cells, as IL-4 transcripts were present in the  $\gamma\delta$  T cell depleted population. Unfortunately, it was not possible to demonstrate this definitively because of the problems associated with preparing RNA from cells that were positively selected using MAbs to the TCR complex. In repeat experiments RNA could not be recovered from CD3<sup>+</sup> cells and the recovery of RNA from  $\alpha\beta$ <sup>+</sup> and  $\gamma\delta$ <sup>+</sup> cells was inconsistent. In addition, the presence of IL-2 transcripts, that are not expressed in unseparated LN cells at 24 h p.i. with L3, in the positively selected  $\alpha\beta$  and  $\gamma\delta$  cells populations, was indicative of T cell activation. The reason for this particular problem is unclear, since all the cell selections were performed on ice or at 4°C to prevent cell activation and PCD. Although the data indicate that the early, IL-4 producing cells are DN  $\alpha\beta$  T cells, definitive proof would require a protocol that selects without activating  $\alpha\beta$ <sup>+</sup> cells.

In addition, it would be interesting to further define whether these cells belong to the unconventional T cell subpopulation bearing the NK1.1 marker which are known to promptly produce large amounts of cytokines, in particular, IL-4. This population consists of IL-4 producing NK1.1<sup>+</sup> CD4<sup>+</sup> (Yoshimoto and Paul, 1994; Emoto *et al*, 1995), CD4<sup>-</sup> CD8<sup>-</sup>  $\alpha\beta$  T cells (Zlotnik *et al*, 1992), and NK1.1<sup>+</sup>  $\gamma\delta$  T cells (Vicari *et al*, 1996). The majority of  $\alpha\beta$ <sup>+</sup> NK 1.1<sup>+</sup> cells are positively selected on class I or class I-related molecules and have restricted TCR chain usage (MacDonald, 1995). The best characterised of the NK1.1<sup>+</sup> population, in terms of early cytokine production, is the CD4<sup>+</sup> subset which secretes IL-4 in the spleen within 90 min of i.v. injection of anti-CD3 antibody or with the superantigen staphylococcal enterotoxin B (Yoshimoto and

Paul, 1994). Interestingly, injection of anti-CD3 in the footpad results in considerable IL-4 mRNA in the popLN after 90 min, with levels remaining elevated for 24h (Yoshimoto and Paul, 1994). Furthermore, in an attempt to formally prove a role for NK1.1<sup>+</sup> cells in Th2 induction, it has recently been shown that splenic CD4<sup>+</sup> NK1.1<sup>+</sup> T cells are essential to restore the ability of  $\beta 2M^{-/-}$  mice to induce IL-4 and produce IgE in response to anti-IgD (Yoshimoto *et al*, 1995) and that their absence in SJL mice is associated with the defect in IgE and IL-4 production in this strain (Yoshimoto *et al*, 1995a). In addition, "conventional" T cells that become primed for IL-4 production *in vivo* could acquire the expression of NK1.1, just as they regulate expression of known activation markers. Gollob and Coffman (1994) described a minority subpopulation of IL-4 secreting memory-like CD4<sup>+</sup> cells, generated by *in vitro* stimulation with anti-CD3, that are capable of directing Th2 development *in vivo*. These cells have a CD62L<sup>low</sup> phenotype as do NK1.1<sup>+</sup> T cells. However, it is not thought that NK1.1 is a universal marker for T cells that may induce Th2 responses by their early production of IL-4. In a recent study it was shown that the burst of IL-4 mRNA expression in the spleens of susceptible mice 90 min after i.v. injection with *L. major* is produced by a CD4<sup>+</sup> NK1.1<sup>-</sup> population that also does not exhibit the V $\beta$  restriction typical of the NK1.1 population (Launois *et al*, 1995). However, it is also unlikely that this population is a cross-reactive memory type CD4<sup>+</sup> cell since IL-12 down-regulates its IL-4 expression and it is known that IL-12 has no effect on fully differentiated Th2 cells (Wang *et al*, 1994). Interestingly, this study also showed that injection of anti-CD3 antibodies can induce expression of IL-4 in CD4<sup>-</sup> T cells. In addition, DN  $\alpha\beta$  T cells are induced in response to infectious agents such as *Listeria monocytogenes* and although these cells display the typical skewed V $\beta$  repertoire, the majority do not bear the NK1.1 marker (Matsuzaki *et al*, 1995).

As BALB/c mice do not express the NK 1.1 marker, it was not possible to confirm whether the DN T cell identified in this study was NK 1.1<sup>+</sup>. Preliminary studies in C57BL/6 mice (which express NK 1.1) and in BALB/c mice demonstrate that IL-4 is

induced in the spleen of both strains within 6 h of i.v. injection of L3. In future experiments, it should be possible to determine if the IL-4 producing cells in the spleen are NK1.1 cells and/or display restricted V $\beta$  expression. Since NK1.1<sup>+</sup> T cells are a minor population in secondary lymphoid organs, using the spleen, rather than popLN, should allow sufficient numbers of cells to be purified for analysis. Furthermore, s.c. infection with L3 induces an almost identical Th2 response at day 12 by splenocytes from C57BL/6 and BALB/c mice. C57BL/6 mice are known to mount a protective Th1 response to *L. major*, while a Th2 response and disease develops in BALB/c mice. Since *Leishmania* promastigotes are not thought to be potent Th polarizers, this differential response is thought to reflect inherent Th phenotype susceptibilities of these mouse strains. Therefore, the capacity of L3 to induce a Th2 polarized response in C57BL/6 mice is consistent with the L3 as a strong Th2 stimulus and gives further support to the early IL-4 burst being an essential contributor to the subsequent Th2 dominated response.

While it has been postulated that prototypic Th2 agents, for example allergens and nematode parasites, may have associated superantigens that are able to activate a sufficient number of NK1.1<sup>+</sup> T cells (Yoshimoto and Paul, 1994), this has yet to be proven. The variety of early IL-4 sources that have been reported only recently, following challenge with different antigenic stimuli, emphasizes the fact that both antigen and route of infection or immunisation will influence the cell type that is activated. This is illustrated clearly when considering the response to three different helminth parasites, characterised as a group as being potent Th2 inducers. The early IL-4 producer in response to s.c. infection with the L3 of *B. pahangi* in this study is likely to be a DN  $\alpha/\beta$  T cell, while mast cells, basophils or eosinophils and  $\gamma/\delta$  T cells are responsible following i.p. infection with *S. mansoni* eggs (Sabin and Pearce, 1995) and L3 of *N. brasiliensis* (Ferrick *et al*, 1995), respectively.

This early kinetic analysis of cytokine gene expression has allowed the mechanism underlying the Th2 polarised response elicited by L3 to be clarified. The infection in humans is similarly characterised by a dominant Th2 response and the down-regulation of antigen-specific Th1 responses. A variety of mechanisms have been proposed to explain the Th2 bias in chronically infected humans, including parasite-induced Th1 anergy (Maizels and Lawrence, 1991), thymic or peripheral Th1 deletion (Steel 1994), or the preferential expansion of Th2 cells (King *et al*, 1993; Maizels *et al*, 1995). In this study, the rapid induction of IL-2 mRNA transcription by both L3 and mf, peaking at day 4 and returning to baseline levels by day 7 p.i., appears to rule out a mechanism of early Th1 anergy. Anergy is classically associated with a block in IL-2 gene transcription (Schwartz, 1990) and cytokine production in normal cells is tightly regulated at the transcription level (Brorson *et al*, 1991). However, an uncoupling of cytokine mRNA expression and protein secretion during the induction phase of T cell anergy has been described by Schall *et al* (1992), who suggested that anergy-specific post-transcriptional mechanisms may exist by which cytokine release is regulated. As the kinetics of IL-2 mRNA induction following infection with L3 was almost identical to that of the Th1-inducing mf and our data, for all the cytokines measured, showed a tight correlation between the pattern of mRNA expression and protein production, it seems unlikely that Th1 cells are anergised due to a lack of IL-2 production immediately upon encounter with the L3. The presence of IL-2 transcripts at day 4 p.i. was not unexpected in view of its role, with IL-4, in stimulating Th2 expansion (Le Gros *et al*, 1990; Abehsira-Amar *et al*, 1992). However, these experiments deal only with a limited number of time points early after infection, and it cannot be ruled out, on this data alone, that anergic mechanisms may be involved in maintaining the antigen-specific Th1 cell unresponsive state.

Although the L3 normally moults to the L4 around day 6-7 p.i., there was no evidence of any Th1 activity preceding this time point, a finding which also rules out a potential Th1→Th2 switch stimulated by developing stages of the parasite. In murine



schistosomiasis, a switch to a Th2 phenotype is well documented at egg laying (Pearce *et al*, 1991). In this study, IL-4 and IL-10 mRNA levels were elevated at day 4 and day 7 p.i., in the absence of any IFN- $\gamma$  induction, with CD4<sup>+</sup> cells contributing to both IL-4 and IL-10 transcription at day 4. This data would suggest that the eventual highly polarised Th2 response measured at day 10 p.i. results from the preferential activation of Th2 cells from the outset of infection, in the absence of Th1 priming. This appears to be at odds with the demonstration that antigen-specific Th1 cytokine responses can be detected, when IL-10 is neutralised (as described in Chapter 4). However, a unifying explanation is that following infection with L3, antigen-reactive Th1 cells are not primed *in vivo* in the presence of IL-10, but that neutralisation of IL-10 allows primed, but uncommitted Th or Th0 cells to express IL-2 and/or IFN- $\gamma$  when re-stimulated with antigen *in vitro*. Clearly further experiments, perhaps involving a more extensive early time course, are required to establish whether antigen-reactive Th1 or Th0 cells can be detected *in vivo* and to elucidate the role of IL-10 in suppressing their Th1 activities. Perhaps IL-10, that is known to induce anergy in Th1 clones by reducing IL-12 and B7 expression by APCs (Kubin *et al*, 1994), may use a similar tolerizing mechanism to block the proliferation and function activity of antigen-specific Th1 cells following infection with L3. Despite these questions, the results of the present experiments indicate that the early responses *in vivo* is dominated by Th2-type cytokines. In this respect, the discovery of a DN T cell population that produces IL-4 in response to the L3 provides the first step in the pathway leading to the differentiation of Th2 cells.

The nature of the stage-specific L3 antigen(s) capable of triggering IL-4 production is obviously of great interest. Both  $\gamma\delta$  and NK1.1<sup>+</sup> DN T cells have been described that recognise the MHC I-related, non-polymorphic molecule CD1 (Porcelli *et al*, 1992; Bendelac *et al*, 1995). The restricted antigen reactivity of these cells suggests that they may function as prompt responders (Bendelac, 1995a). In humans, CD1 seems to present lipid rather than peptide antigens to T cells. For example, CD1-restricted DN T cell lines specific for the mycobacterial lipids, mycolic acid and lipoarabinomannan

have been described (Beckman *et al*, 1994; Sieling *et al*, 1995). Moreover, it has been proposed that CD1-restricted DN T cells derived from the skin lesions of leprosy patients are an important factor in determining the Th cell response to *Mycobacterium leprae* via their specific cytokine production (Sieling *et al*, 1995). In this respect, it is interesting to note that the surface of the L3 worm, which in part consists of lipid-containing molecules (Proudfoot *et al*, 1991), rapidly turns-over within 24h of entry into the mammalian host (Carlow *et al*, 1987)

Many of the human CD1-restricted DN T cell lines and also CD1-specific T cells from normal unimmunised mice appear to be auto-reactive, in that they recognise CD1<sup>+</sup> cells without foreign antigen. It has been suggested that the upregulation of CD1 expression alone or in association with self-peptide induced by the stress of inflammation and/or infection may constitute an internal "danger" signal that then promotes anti-inflammatory responses through the early secretion of IL-4 (Bendelac *et al*, 1995a). Histological sections taken at the site of injection with L3 reveal areas of oedematous and inflamed tissue in the infected footpad with prominent dilated lymphatic vessels containing developing larvae (data not shown). Whether the DN T cell is reactive against L3-specific antigens or self-antigens (that are expressed upon stress-induced cell activation), this cell type could represent a form of innate immunity that, through its prompt production of IL-4, may play a central role in the Th2 bias in filarial infection.

## **CHAPTER 6**

## **Chapter 6 Infection with mf induces apoptosis of splenic T cells: a mechanism for antigen-specific proliferative unresponsiveness?**

### **6.1. Introduction**

The aim of this chapter was to investigate whether the antigen-specific proliferative unresponsiveness of spleen cells from mf-infected BALB/c mice, observed in Chapter 3, could be attributed to the induction of T cell apoptosis. The rationale for these experiments was based on the fact that spleen cells from mice infected with  $10^5$  mf, were unable to proliferate in response to filarial antigen after 3 days in culture and exhibited a mean S. I.  $<1$  after 4 days. Apoptosis has been associated with proliferative unresponsiveness and reduced cell viability in lymphocyte cultures, following TCR stimulation, in other models of infectious diseases (see below). Furthermore, this proliferative response is exclusive to infection with  $1 \times 10^5$  mf, since spleen cells from L3-infected mice exhibited substantial antigen-stimulated proliferation after 3 days in culture and spleen cells from mice infected with L3,  $1 \times 10^4$  mf or from uninfected mice did not exhibit a mean S.I.  $< 1$  after 4 days in culture with antigen.

It is now obvious that multiple pathways mediate apoptosis of mature T cells, as mentioned in the Introduction. However, it has been further suggested that apoptosis of mature T cells could be classified into two types depending on their activation state (Van Parijs 1996). PCD is undergone by naive T cells following growth factor deprivation or inadequate stimulation, and can be blocked by anti-CD28 costimulation, the presence of growth factors like IL-2 and activation of survival-associated genes like bcl-2 and bcl-X<sub>L</sub> (Boise *et al*, 1995; Van Parijs *et al*, 1996). AICD occurs as a result of repeated TCR stimulation and has been shown to be dependent on Fas, TNF-R, or Nur77 ligation (Woronicz *et al*, 1994; Brunner *et al*, 1995; Zheng *et al*, 1995). Unlike PCD, Fas-mediated AICD is not prevented by CD28-mediated costimulation or the

expression of bcl-2 and bcl-X<sub>L</sub>, and is actually augmented by IL-2 (Van Parijs *et al*, 1996; Strasser *et al*, 1995).

Several methods are now available to measure apoptotic cells based upon the detection of DNA cleavage. In lymphocytes, as in other cell types, apoptosis is accompanied by the activation of endogenous endonucleases leading to the internucleosomal cleavage of DNA (Cory, 1994). Therefore, partial digestion results in a DNA ladder, that can be visualised following gel electrophoresis as multimers of approximately 200bp. Another method involves the FACS analysis of PI stained cells, which defines the proportion of apoptotic lymphocytes within a sample as those with subdiploid DNA content. Finally, early apoptotic cells can be visualised *in situ* in tissue sections by labelling nicked DNA. DNA laddering, reduction of the total DNA content below 2N, and detection of single stranded DNA breaks are now considered to be as much hallmarks of apoptosis as the characteristic morphological changes.

Many of the early experiments describing T cell apoptosis used chronic stimulation of the TCR, with anti-TCR antibodies or SAg, or the equally artificial system of TCR Tg mice in which the majority of the T cells, being TCR Tg<sup>+</sup>, are then activated with their specific antigen to induce apoptosis (as referenced in 1.5). Understandably, it has been more difficult to directly detect apoptotic death with nominal antigen in normal mice. For example, apoptosis was suggested as a mechanism of T cell deletion following exposure to high concentration of antigen (myelin basic protein), based only upon the reduction in cell viability observed after *in vitro* stimulation (Critchfield *et al*, 1994). However, the development of such sensitive techniques, described above, has just recently permitted T cell apoptosis to be demonstrated in the peripheral deletion of antigen-reactive T cells in orally tolerised, normal mice (Garside *et al*, 1996). In that study, tolerised lymphocytes from mice fed with OVA, that were unable to proliferate in response to antigen *in vitro*, displayed enhanced mortality, when cultured in the absence of antigen, in comparison to cells from immunised control animals. Detection

of hypodiploid cells by PI staining combined with the examination of lymphocyte morphology suggested that both CD4<sup>+</sup> and CD8<sup>+</sup> cells from tolerant mice were dying by apoptosis.

Furthermore, host cell apoptosis has been revealed in several models of infectious diseases. It has been suggested that mature T cell apoptosis could play a role in immunosuppression caused by protozoal, bacterial and viral infections. For example, during acute infection with *Trypanosoma cruzi*, there was a striking reduction in the numbers of CD4<sup>+</sup>, but not CD8<sup>+</sup>, T cells in the spleen. This correlated with accelerated death and DNA fragmentation in cultures of CD4<sup>+</sup>, but not CD8<sup>+</sup>, spleen cells stimulated with anti-TCR $\alpha\beta$  and anti-CD3. Addition of rIL-2 potentiated this effect (Lopes *et al*, 1995; Lopes and DosReis *et al*, 1996). Furthermore, splenic T cells from infected mice were markedly unresponsive to anti-TCR $\alpha\beta$  and anti-CD3 in proliferative assays. To demonstrate that apoptosis was not simply an *in vitro* phenomenon, it was then shown that DNA fragmentation could be detected in freshly isolated splenocytes from infected, but not uninfected mice (Lopes *et al*, 1995). In addition, DNA fragmentation was observed in freshly isolated spleen cells from acutely infected, but not uninfected mice, following injection of mice with anti-CD3 (Lopes and DosReis 1996).

Similarly, it was found that spleen cells from mice acutely infected with lymphocytic choriomeningitis virus (LCMV) displayed impaired proliferation with ConA and anti-CD3 and die rapidly by apoptosis as assessed using FACS analysis of PI stained cells (Razvi and Welsh, 1993). In addition, pre-culture of T cells, from acutely infected mice, with IL-2 accelerated apoptosis upon subsequent anti-CD3 crosslinking. These results may be important in relation to the observation that LCMV deletes anti-viral cytotoxic effector T cells resulting not only in virus persistence and reduced recall responses but also the elimination of lethal immunopathology (Moskophidis *et al*, 1993). It has also been shown that patients with viral infections (varicella zoster virus and EBV) possess

circulating activated CD45RO<sup>+</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells with low bcl-2 expression that rapidly undergo apoptosis in culture (Akbar *et al*, 1993). However, in contrast to the findings from the murine LCMV model, apoptosis of CD45RO<sup>+</sup> T cells from virally infected human donors can be prevented by addition of IL-2 which results in a concomitant increase in bcl-2 expression (Akbar *et al*, 1993). HIV infection in humans is characterised by a number of disturbed T cell functions including impaired proliferation and increased apoptosis of CD4<sup>+</sup> T cells following TCR and mitogen stimulation (Groux *et al*, 1992). Furthermore, addition of anti-CD28 to cultures, from HIV-infected asymptomatic individuals, prevented CD4<sup>+</sup> T cell apoptosis and restored the defective T cell proliferative responses. Cell lines infected with HIV express Fas and are susceptible to anti-Fas MAb induced death (Kobayashi *et al*, 1990), although the *in vivo* significance of Fas antigen in AIDS pathogenesis remains to be elucidated. One suggestion is that bacterial infections may accelerate progression from HIV-positive to AIDS by augmenting Fas-mediated apoptosis of CD4<sup>+</sup> T cells. This followed the observation that infection with a retrovirus that causes murine AIDS induces Fas-positive CD4<sup>+</sup> T cells, which rapidly undergo apoptosis when stimulated via Fas, and are increased in number when mice are concomitantly infected with *Mycobacterium avium* (Hiromatsu *et al*, 1994).

In another model, infection of mice with *Listeria monocytogenes* was shown to induce apoptosis of infected hepatocytes with the release of neutrophil chemottractants resulting in microabcess formation and neutrophil-killing of *Listeria* within the apoptotic cells (Rogers *et al*, 1996). However, using neutrophil-depleted mice, it was shown that hepatocyte apoptosis and the associated recruitment of neutrophils, during the early phase of infection, was necessary for the subsequent development of acquired T cell immunity to *Listeria*.

Therefore, there seems to be no unifying explanation as to why infection with intracellular agents is associated with apoptosis of host cells; it appears to depend on

the pathogen and the host cell(s) involved. This would suggest that different mechanisms are being evoked by the various intracellular agents. Evidence for both PCD and AICD of lymphocytes is apparent in different infectious disease models, although little has been done yet to characterise the pathways. One exception is cowpox virus that has been shown to encode for a soluble form of the TNF-R (Smith *et al*, 1994) and CrmA, the protease inhibitor that blocks Fas-transduced apoptosis by binding to ICE (Enari *et al*, 1995; Los *et al*, 1995). It seems likely, therefore, that the cowpox virus may be endeavouring to prevent the suicide of infected host cells until the cell ruptures when sufficient viral replication has taken place (Ray *et al*, 1992).

The aim of the experiments in this chapter were to determine whether apoptotic cells could be detected in spleens from mice infected with  $10^5$  mf, using the methods described above. Parallel analysis was also performed on spleen cells from mice infected with L3, to ensure that apoptosis is not induced by infection *per se*, and with lower numbers of mf ( $1 \times 10^4$  and  $1 \times 10^3$ ) to determine whether a dose effect could be revealed. Attempts were also made to investigate the mechanism of apoptosis in this model and to determine whether it reflects AICD or PCD.



## **6.2. Results**

### **6.2.1. Detection of apoptotic cells in spleens of mice infected s.c. with mf but not L3**

In this experiment, groups of three mice were injected s.c. with  $1 \times 10^5$  mf, 50 L3 or HBSS (con). Spleen cell suspensions were prepared as described before, and freshly isolated spleen cells from each group were stained immediately with PI or frozen as pellets for DNA extraction. The remaining spleen cells were cultured, under the same conditions that were used in proliferation assays; (duplicate) 200  $\mu$ l cultures (at  $5 \times 10^6$  cells/ml) with or without antigen (10  $\mu$ g/ml). Proliferation assay of spleen cells from mice infected with  $1 \times 10^5$  mf, but not 50 L3 or  $1 \times 10^4$  mf, had revealed that the presence of antigen was associated with cell death (S.I.<1 at 4d) (Chapter 3). Therefore, cell death was determined over time in culture by trypan blue exclusion, PI staining and DNA fragmentation. The time points in culture (24h, 96h and 120h) were chosen to cover the incubation times measured in the proliferation assays (88h, 112h). The FACS analysis gates only PI stained lymphocytes and discriminates at the level of the DNA content/per cell between single lymphocytes and, for example, two lymphocytes that are stuck together or that are just about to separate after mitosis.

#### **Trypan blue exclusion**

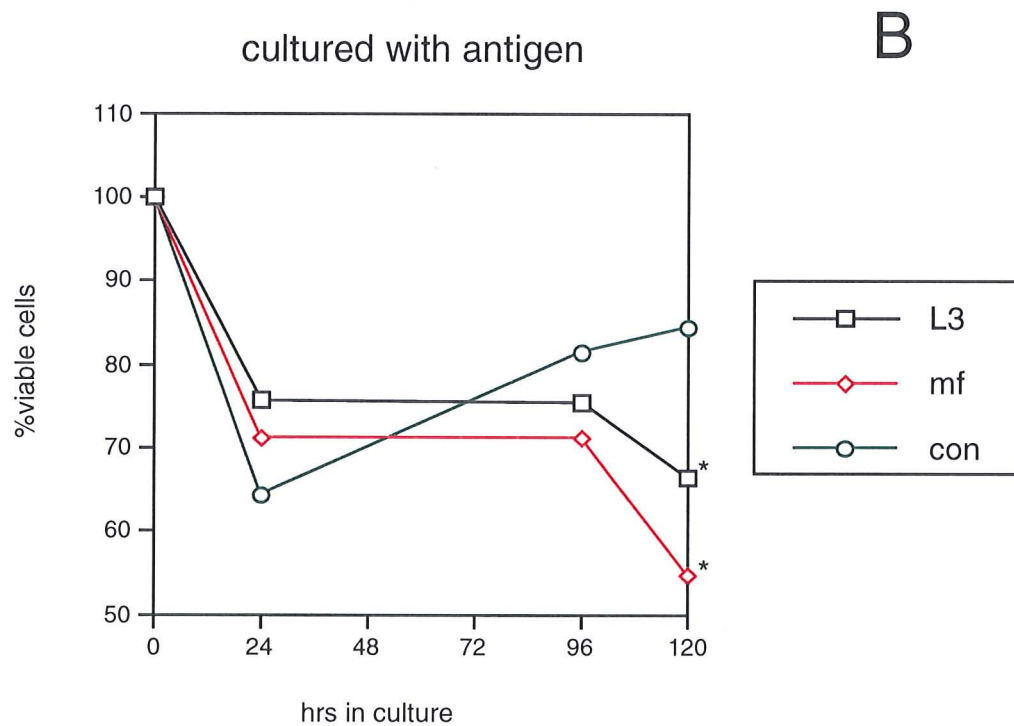
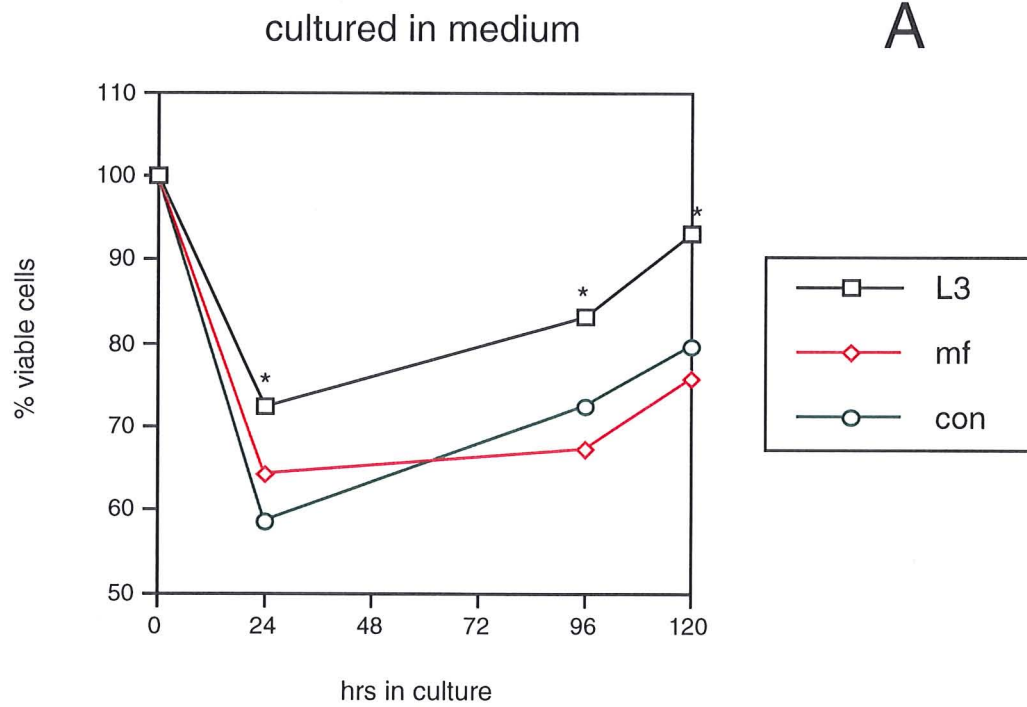
The number of viable spleen cells from the three groups of mice, remaining in culture over time, in the presence or absence of antigen, was assessed by trypan blue exclusion. Fig 1 shows the % of viable cells in medium only (A) and antigen stimulated cultures (B) over time.

Two very distinctive patterns in cell survival were apparent dependent firstly, on whether the spleen cells were from infected or uninfected mice and secondly, on whether cells from infected mice were cultured in the presence or absence of antigen.

**FIGURE 6.1. Viability of spleen cells in cultures from BALB/c mice injected s.c. with 50 L3,  $1 \times 10^5$  mf or HBSS**

Three BALB/c mice per group were injected s.c. with 50 L3,  $1 \times 10^5$  mf or HBSS (con). At day 12 p.i. spleen cells were cultured in duplicate wells for 24, 96 and 120h in the absence (A) or presence of  $10 \mu\text{g/ml}$  antigen. Results shown are the mean percentages of viable cells remaining in duplicate cultures  $\pm$ SD, as determined by trypan blue staining.

\* Significant difference ( $p = 0.05$ ) between the percentage of viable spleen cells in cultures from infected mice compared to uninfected controls.



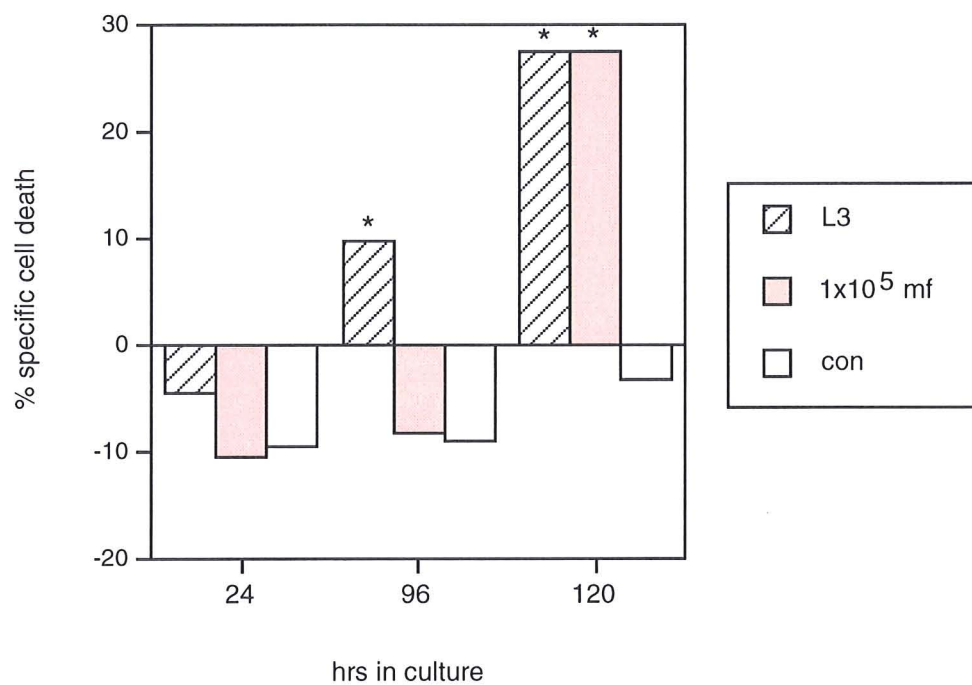
No significant difference could be detected in the % of dead cells from mf-infected mice compared to cells from L3-infected mice.

In the absence of antigen, the pattern of survival of spleen cells from all groups of mice was very similar; after an initial decline in viability, to the lowest point at 24h, there was a steady increase until the last time point measured, 120h (Fig 6.1A). However, there was significantly less death in spleen cell cultures from L3-infected mice compared with cells from uninfected mice, at all time points, in the absence of antigen (Fig 6.1A) (% viable cells at 24h L3=72.5±4.7% versus con=58.6±3.1%; at 96h L3=83.2±4.6% versus con=72.4±7.1%; at 120h L3=93.0±0.8% versus con=79.7±10.3%,  $p = 0.050$  in all cases). No difference was observed in cell viability in the medium-only cultures of spleen cells from mf-infected mice compared to cells from uninfected mice (Fig 6.1A).

Furthermore, the pattern of survival of spleen cells from uninfected controls did not differ significantly if cultured in the presence or absence of antigen (Fig. 6.1A & B). In contrast, in antigen-stimulated cultures of spleen cells from mf-infected mice, there was a rapid decline in cell viability to the lowest point (24h) after which time the level stabilised, before declining again by 120h (Fig 6.1B). Antigen-stimulated cells from L3-infected mice responded in a similar manner (Fig 6.1B). After 120h in culture, spleen cells from both groups of infected mice had significantly reduced survival compared to cells from uninfected mice (% viable cells at 120h mf=54.8±7.9% and L3=66.5±3.3% compared to con=84.3±2.3%,  $p = 0.050$  in both cases).

Since there appeared to be a relationship between death of cells from infected animals and the presence of antigen in culture, statistical analysis was performed on the levels of viability expressed as % specific cell death.

$$\% \text{ specific cell death} = \frac{100 \times (\% \text{ dead in the presence of antigen} - \% \text{ dead in medium})}{100\% - \% \text{ dead in medium}}$$



**FIGURE 6.2. Measurement of specific cell death of spleen cells from BALB/c mice infected with L3 or  $1 \times 10^5$  mf by culture in the presence of antigen.**

Data shown are percentages of specific cell death stimulated by culture in the presence of antigen, as calculated from the results of Fig 6.1 (See Expt 6.2.1).

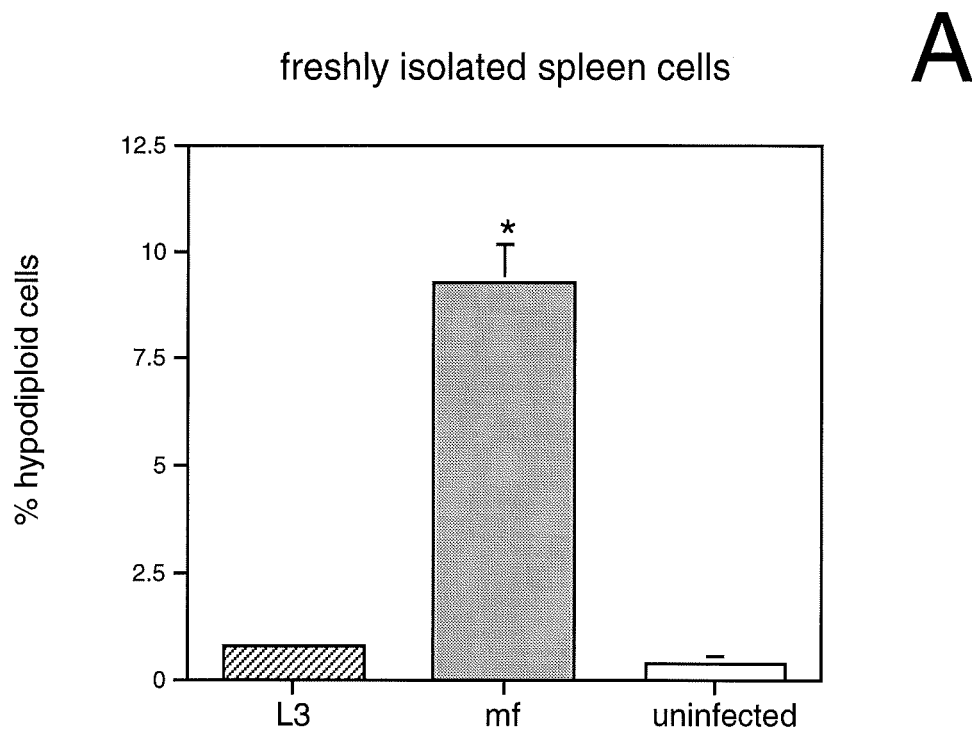
\* Significant difference ( $p < 0.05$ ) between the percentage of specific cell death in cultures from infected mice compared to uninfected controls.

Conversion of % cell viability into % specific cell death would also account for any differences in the change in total cell numbers during culture between the groups. However, despite the fact that previous proliferation data shows spleen cells from L3-infected mice divide in response to antigen while cells from mf-infected mice do not, the pattern in specific cell death in both groups of infected mice were very similar. Fig 6.2 shows that the % specific cell death in antigen-stimulated cultures of spleen cells from mice infected with either L3 or mf was significantly higher at 120h, but not at earlier time points, compared to uninfected control spleen cell cultures (At 120h mf=27.4±9.4%, L3=27.4±2.4% versus con=-3.7±22.5%,  $p = 0.050$  in both cases). Using this method, specific cell death was not measured in cultures from uninfected mice at any time point.

### PI staining and FACS analysis

To examine the basis of the cell loss in antigen-stimulated cultures, freshly isolated and cultured spleen cells from the groups of 3 mice were assessed for hypodiploid DNA content by PI staining and FACS analysis (Fig 6.3). The most obvious difference between mf-infected animals and the other two groups was observed when PI staining was performed on freshly isolated spleen cells (approximately 2h *ex vivo*). Fig 6.3A shows that freshly isolated spleen cells from mice infected with  $10^5$  mf contain a higher proportion of cells with a hypodiploid DNA content compared to freshly isolated spleen cells from L3-infected or uninfected mice (mf=9.3±0.9% compared to L3=0.8±0.1% and con=0.4±0.2%,  $p = 0.05$  in both cases).

However, cultures from all groups, incubated in medium only (Fig 6.3B), contained a comparable high proportion of apoptotic cells after 24h (L3=31.6±1.1%, mf=33.8±3.1% and con=32.9±0.8%), that decreased significantly by 120h (L3=11.8%, mf=17.6% and con=12.2%,  $p = 0.050$  compared to 24h). The % of apoptotic cells in cultures from L3-infected and uninfected mice had also decreased by 96h compared to 24h ( $p=0.050$  in both cases), in contrast to the level in cultures from mf-infected mice

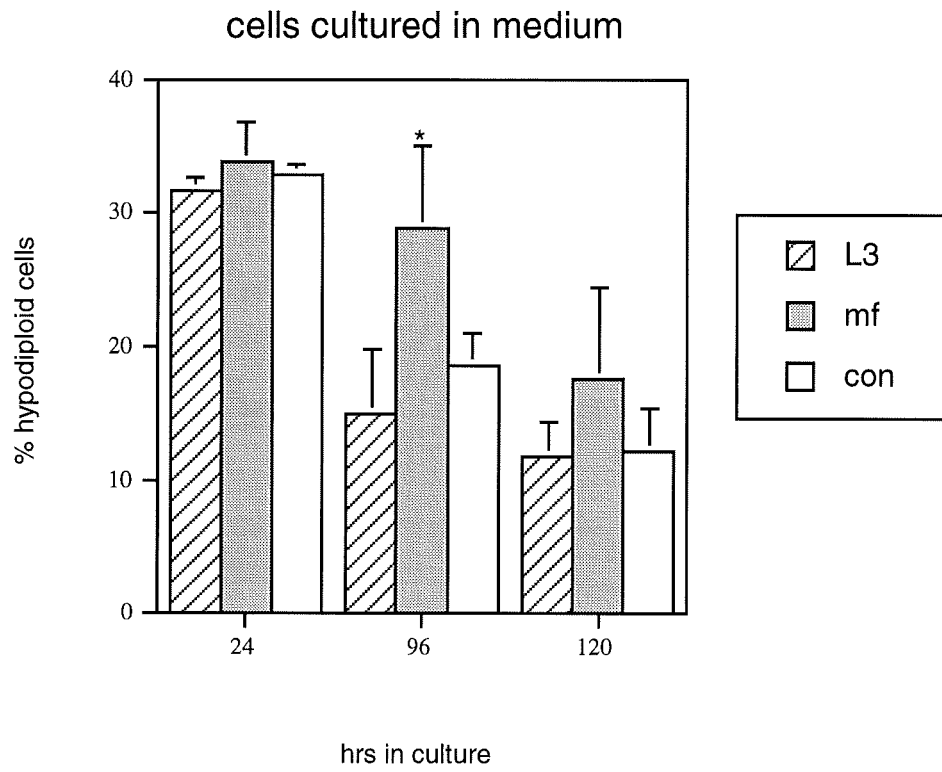


**FIGURE 6.3. Detection of apoptosis in spleen cells from BALB/c mice injected s.c. with 50 L3,  $1 \times 10^5$  mf or HBSS by PI staining**

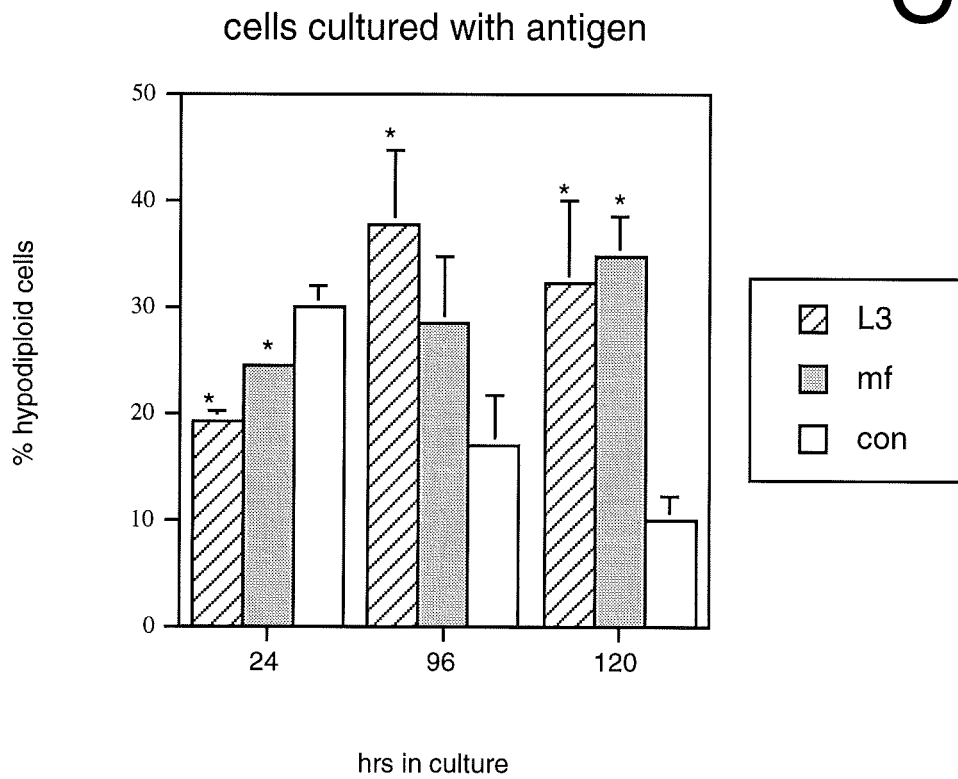
BALB/c mice were injected as described in the legend to Fig. 6.1. Freshly isolated spleen cells (A) and spleen cells cultured for 24, 96 and 120h in medium (B) or in the presence of  $10 \mu\text{g/ml}$  antigen (C) were stained with PI and flow cytometry performed as described in 2.4.3. Data shown are the percentage of hypodiploid (apoptotic) cells in the each sample  $\pm$  SD. \* Significant difference ( $p < 0.05$ ) between the percentage of hypodiploid spleen cells from infected mice compared to uninfected controls.

Figure 6.3.

B



C





that remained significantly higher than the other two groups (At 96h mf=28.9% compared to L3=15.1% and con=18.7%,  $p = 0.05$  in both cases).

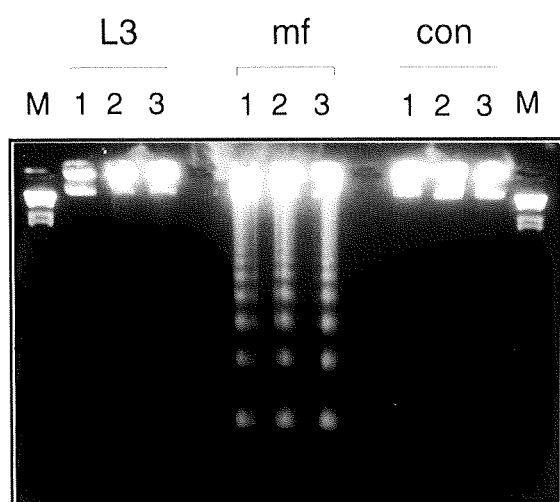
The % of apoptotic cells in antigen-stimulated cultures from the different groups was more divergent (Fig 6.3C). Like the medium-only cultures, there was a substantial number of apoptotic cells in antigen-stimulated cultures from all groups after 24h. However, unlike the medium only cultures, the proportion of apoptotic cells was significantly lower in antigen-stimulated cultures of cells from L3- and mf-infected mice compared to uninfected controls (L3=19.3±0.9% and mf=24.5±0.5% compared to con=30.1±2.0%,  $p = 0.05$  in both cases). However, after 96 and 120h of culture in the presence of antigen this pattern had reversed completely. At both time points the % of apoptotic cells in antigen-stimulated cultures from L3- and mf-infected mice was significantly higher compared to uninfected controls (96h, L3=37.7% and mf=28.4% compared to con=17.1%,  $p = 0.05$  in both cases; 120h, L3=32.3% and mf=34.7% compared to con=9.9%,  $p = 0.05$  in both cases).

Furthermore, consistent with the % viability and % specific cell death in these cultures, assessed by trypan blue staining, there appeared to be a positive association between the % of hypodiploid cells detected at the latest time-point (120h) and the presence of antigen in cultures of spleen cells from L3- and mf-infected but not uninfected mice (Fig 6.3B & C). After 120h, the % of apoptotic cells in cultures of spleen cells from L3 and mf-infected mice, but not uninfected mice, was significantly higher in the presence than in the absence of antigen (L3 Ag=32.3±7.6% compared to L3 med=11.8±2.8 and mf Ag=34.7±3.9% compared to mf med=17.6±6.8%,  $p=0.05$  in both cases; con Ag=9.9±2.4% compared to con med=12.2±3.3%,  $p>0.05$ ). In addition, the % of apoptotic cells in antigen-stimulated cultures of spleen cells from L3 and mf-infected mice was significantly greater than in antigen-stimulated cultures of cells from uninfected controls (L3 Ag=32.3±7.6% and mf Ag=34.7±3.9% compared to con Ag=9.9±2.4%,  $p = 0.05$  in both cases).

**FIGURE 6.4. Detection of apoptosis in spleen cells from BALB/c mice injected s.c. with 50 L3,  $1 \times 10^5$  mf or HBSS by DNA fragmentation**

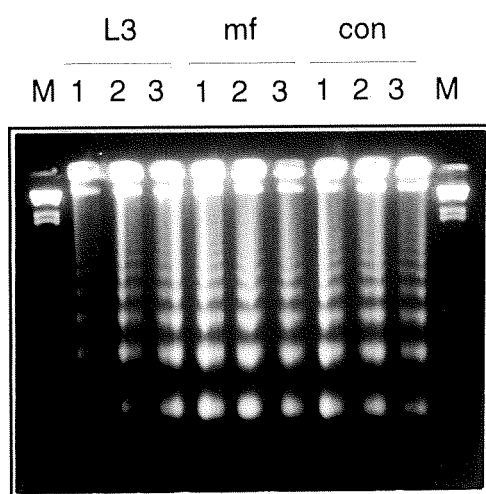
BALB/c mice were injected as described in the legend to Fig. 6.1. Agarose gel electrophoresis was performed using DNA extracted from freshly isolated spleen cells (A) and spleen cells cultured in medium or the presence of  $10 \mu\text{g/ml}$  antigen for 24h (B), 96h (C) and 120h (D). M is the molecular weight markers ( $\lambda$  HindIII). 1, 2 and 3 denotes the three mice per group.

Figure 6.4



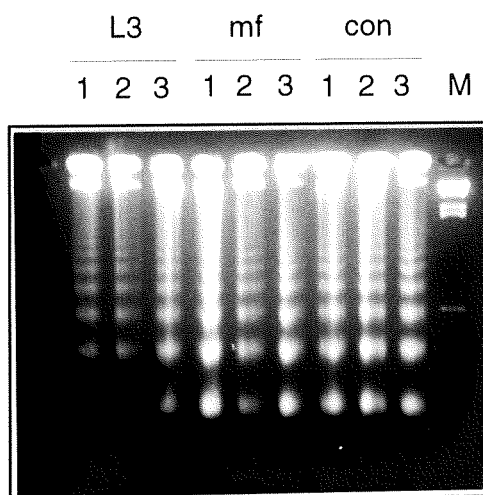
A

freshly isolated



B

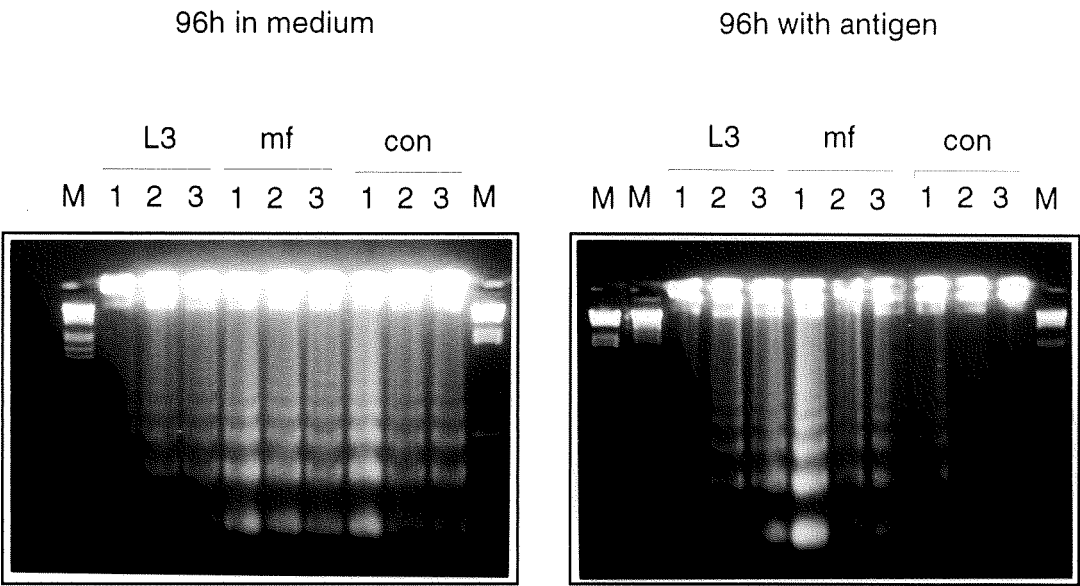
24 h in medium



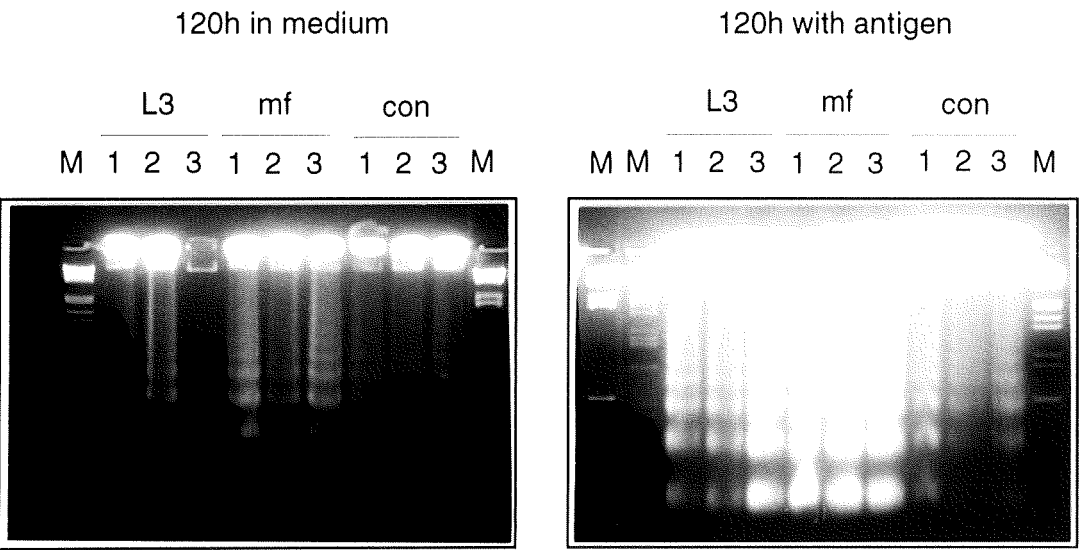
24h with antigen

Figure 6.4

C



D



## DNA fragmentation

DNA was prepared from freshly isolated and cultured cells to detect DNA laddering. Consistent with the PI staining results, DNA fragmentation was observed in samples prepared from freshly isolated spleen cells only from mf-infected mice; DNA prepared from freshly isolated spleen cells from uninfected or L3-infected mice was intact (Fig 6.4A). Furthermore, DNA fragmentation was apparent in medium-only and antigen-stimulated cultures of cells from all 3 groups after 24h (Fig 6.4B), 96h (Fig 6.4C) and 120h (Fig 6.4D).

### 6.2.2 Detection of apoptosis in spleens of mice infected with mf

The aim of this experiment was firstly to confirm the results from Expt. 6.2.1, that apoptotic cells could be detected only in freshly isolated spleen cells from mf-infected mice, and secondly to determine the maximum length of time spleen cells could be cultured before cells from all groups of mice, including uninfected mice, undergo apoptosis. It has been reported previously that mouse splenic T lymphocytes die at a relatively high rate upon explant (Razvi and Welsh, 1993). Groups of three mice were injected s.c. with  $1 \times 10^5$  mf or HBSS (con). Freshly isolated spleen cells (2h *ex vivo*) and spleen cells, that have been *ex vivo* in medium at 37°C for 4h, from both groups of mice, were stained with PI for FACS analysis or frozen before DNA extraction.

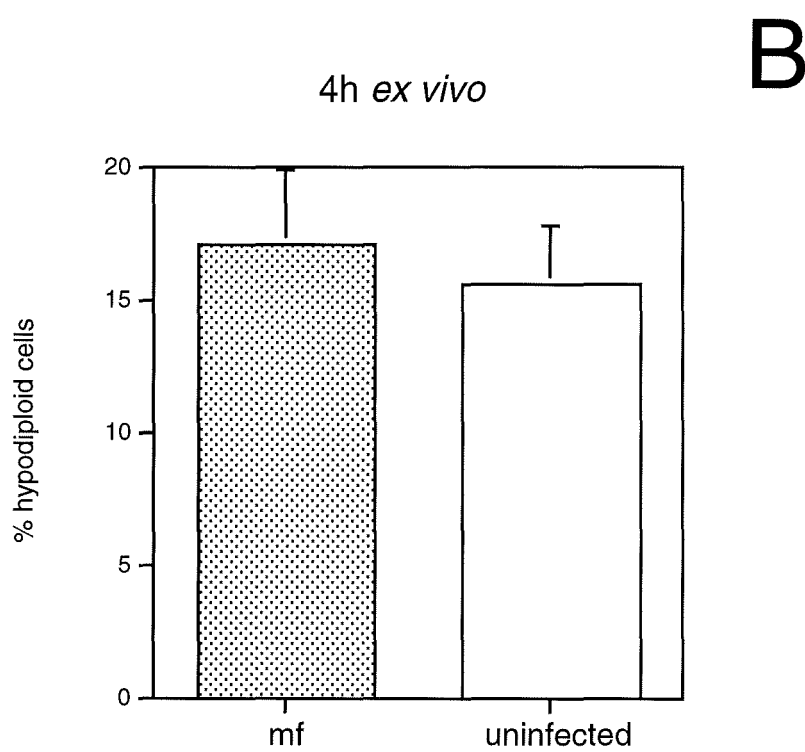
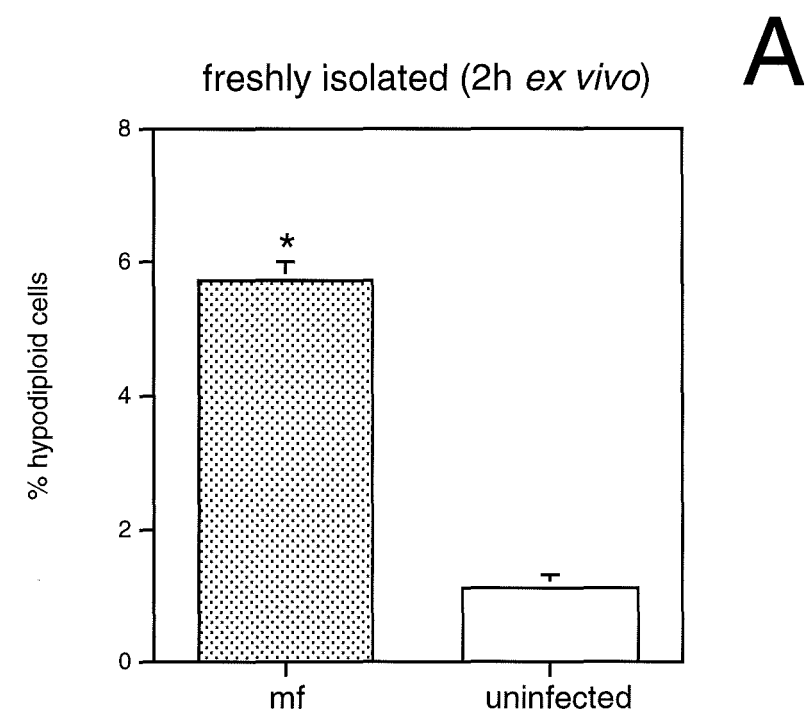
### PI staining and FACS analysis

Fig 6.5A confirms that freshly isolated spleen cells from mice infected with mf contained a significantly higher percentage of cells with a hypodiploid DNA content compared to freshly isolated spleen cells from uninfected mice (mf= $5.7 \pm 0.3\%$  compared to con= $1.1 \pm 0.2\%$ ,  $p = 0.050$ ). In contrast, after a further 2h *ex vivo*, there was an increase in hypodiploid cells in spleen cells samples from both groups of mice to a level that was comparable between the groups (mf= $17.1 \pm 2.8\%$  compared to con= $15.6 \pm 2.2\%$ ,  $p > 0.05$ ) (Fig 6.5B).

**FIGURE 6.5. Detection of apoptosis in spleen cells from BALB/c mice infected with  $1 \times 10^5$  mf by PI staining**

Groups of three BALB/c mice were injected s.c. with  $1 \times 10^5$  mf or HBSS. At day 12 p.i., freshly isolated spleen cells (A) and spleen cells cultured in medium for 4h (B) were stained with PI and flow cytometry performed as described in 2.4.3. Data shown are the percentage of hypodiploid (apoptotic) cells in each sample  $\pm$ SD. \* Significant difference ( $p = 0.05$ ) between the percentage of hypodiploid cells in spleen cell cultures from mf-infected mice compared to uninfected controls.

**Figure 6.5**

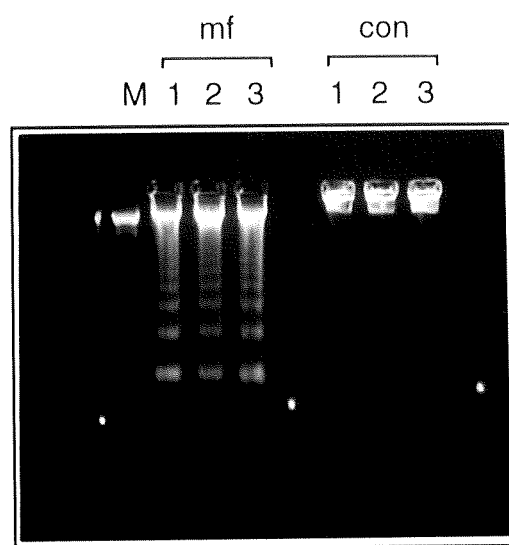


**FIGURE 6.6. Detection of apoptosis in spleen cells from BALB/c mice infected with  $1 \times 10^5$  mf by DNA fragmentation**

Mice were injected as described in the legend to Fig. 6.5. Agarose gel electrophoresis was performed using DNA extracted from freshly isolated spleen cells (A) and spleen cells cultured in medium for 4h (B). M is the molecular weight markers ( $\lambda$  Hind III). 1, 2 and 3 denotes the three mice per group.

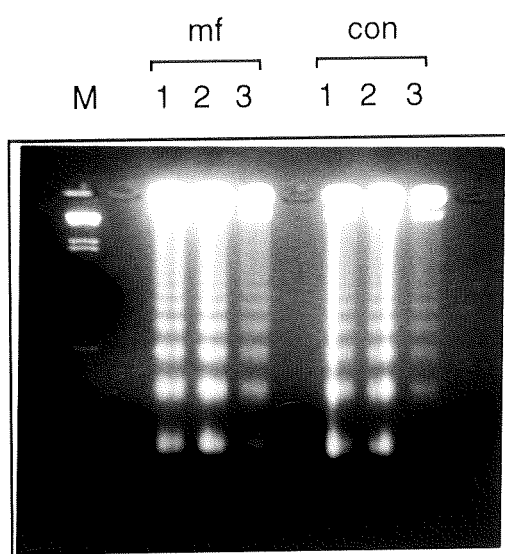


Figure 6.6



A

freshly isolated  
(2h *ex vivo*)



B

4h *ex vivo*

## **DNA fragmentation**

The results of the PI staining and FACS analysis was confirmed by the DNA fragmentation assays (Fig 6.6). In the freshly isolated samples, DNA laddering was detected in spleen cells from mice infected with mf but not in cells from uninfected mice (Fig 6.6A). In contrast, DNA fragmentation was apparent in all cells, from both groups of mice, that had been *ex vivo* for 4h (Fig 6.6B).

### **6.2.3. Spontaneous spleen cell death in culture is inhibited by incubation at 4°C**

Spontaneous cell death, that occurs when splenic T cells are placed in *in vitro* culture, is dependent on active cellular processes and can, therefore, be blocked if cells are incubated over-night at 4°C (Lopes 1995). To confirm that spontaneous cell death is responsible for the substantial level of apoptosis detected in all 24h cultures, as evidenced in Expt. 6.1, spleen cells from mice injected s.c. with  $1 \times 10^5$  mf, 50 L3 or HBSS (con) were incubated overnight at 37°C or 4°C in medium only. Cells were then stained with PI or frozen before DNA extraction.

### **PI staining/FACS analysis and DNA fragmentation**

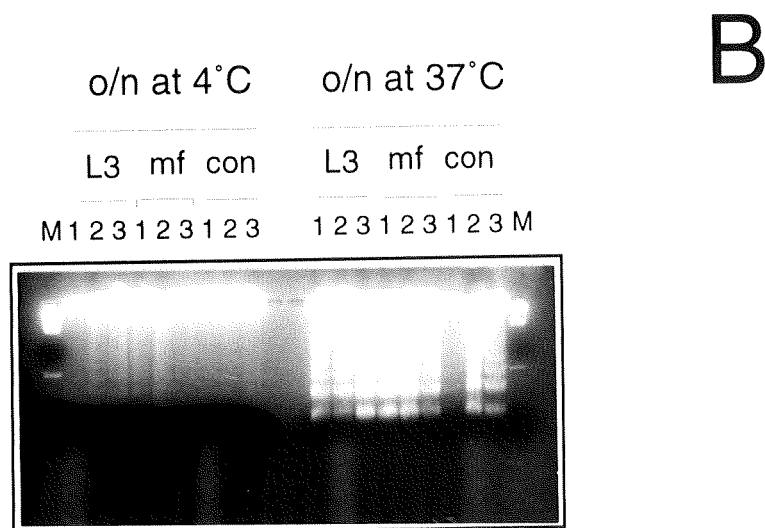
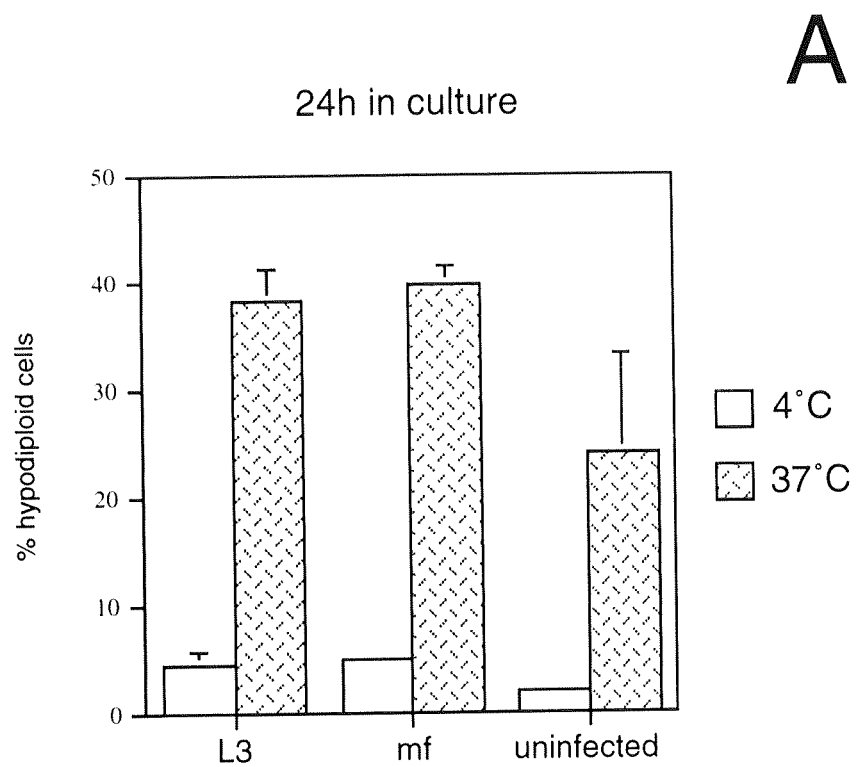
Spleen cells from all groups of mice after 24h in culture at 37°C contained a high proportion of hypodiploid cells by PI staining (Fig 6.7A) and fragmented DNA (Fig 6.7B), as observed previously in Expt. 6.1. However, when the same cells, from all groups, were incubated for 24h at 4°C, the generation of hypodiploid cells was blocked substantially (nearly 10-fold) (Fig 6.7A) and the DNA that was extracted appeared to be completely intact (Fig 6.7B).

Therefore, considering the results from experiments 1-4, only freshly isolated spleen cell samples (maximum of 2h *ex vivo*) were analyzed in the remaining experiments.

**FIGURE 6.7. Comparison of apoptosis in spleen cells from 3 groups of BALB/c mice after over-night culture at 4°C or 37°C by PI staining (A) and DNA fragmentation (B)**

Three BALB/c mice per group were injected s.c. with 50 L3,  $1 \times 10^5$  mf or HBSS (con). At day 12 p.i., spleen cells were cultured in medium over-night at 4°C or 37°C. (A) Cells were stained with PI and flow cytometry was performed as described in Materials and Methods (Chapter 2). Data shown are the percentage of hypodiploid (apoptotic) cells in each sample  $\pm$ SD. \* Significant difference ( $p = 0.05$ ) between the percentage of hypodiploid cells in spleen cell cultures incubated at 4°C compared to cultures of the same cells incubated at 37°C. (B) Agarose gel electrophoresis was performed using DNA extracted from spleen cells that were cultured in medium over-night at 4°C or 37°C. M is the molecular weight markers ( $\lambda$  Hind III). 1, 2 and 3 denotes the three mice per group.

**Figure 6.7**



#### **6.2.4. Detection of apoptotic cells in spleens of mice infected s.c. with varying doses of mf**

In this experiment mice were injected s.c. with varying doses of mf ( $1 \times 10^5$ ,  $1 \times 10^4$ ,  $1 \times 10^3$ ) or HBSS (con). At day 12 p.i. freshly isolated spleen cells were analysed for the presence of apoptotic cells by PI staining or DNA fragmentation.

##### **PI staining/FACS analysis and DNA fragmentation**

Fig 6.8A shows that spleen cells from mice infected with  $1 \times 10^5$  mf, but not  $1 \times 10^4$  or  $1 \times 10^3$  mf, possess a significantly higher number of hypodiploid cells than uninfected controls ( $10^5$ mf= $2.7 \pm 0.2\%$  compared to  $10^4$ mf= $0.7 \pm 0.1\%$ ,  $10^3$ mf= $0.9 \pm 0.1\%$  and con= $0.8 \pm 0.1\%$ ,  $p = 0.05$  in all cases). Despite the fact that the level of apoptosis in the spleen cell samples from mice infected with  $1 \times 10^5$  mf was lower than previously observed (Expts. 6.2.1 and 6.2.2), a clear DNA laddering pattern could be detected in DNA samples prepared from spleen cells of all three mice from this group (Fig 6.8B). In contrast, a very low level of DNA fragmentation could be visualised in samples prepared from spleen cells of two out of three mice infected with  $1 \times 10^4$  mf (mice nos. 1&2). Consistent with the PI staining data, no DNA laddering was evident in spleen cell samples from mice infected with  $1 \times 10^3$  mf or from uninfected controls (Fig 6.8B).

#### **6.2.5. Spleen cells from mice infected with mf proliferate in response to exogenous IL-2 only in the absence of antigen**

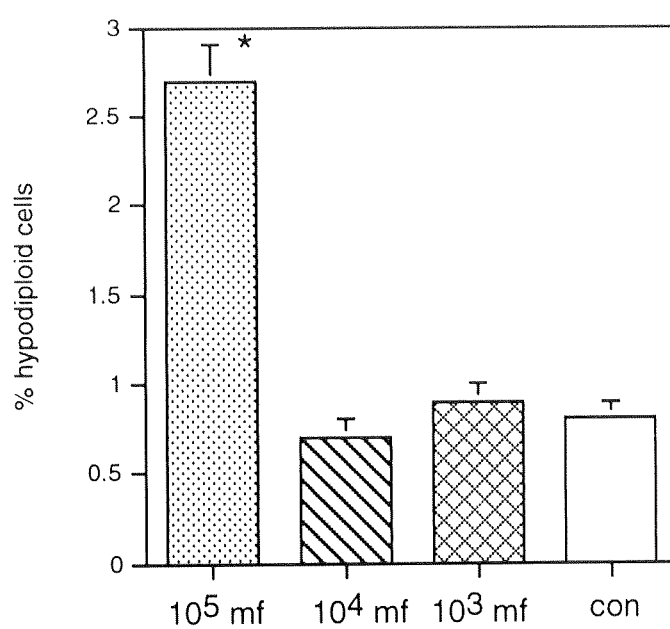
The % specific cell death data from Expt. 6.2.1 revealed an association between the reduced cell viability and presence of antigen in spleen cell cultures from infected mice after 120h. Although no significant difference in % specific cell death could be detected between cultures of spleen cells from mice infected with  $1 \times 10^5$  mf and 50 L3 (Expt. 6.2.1), only spleen cells from mice infected with  $1 \times 10^5$  mf exhibit S.I.<1 after 88h in an antigen proliferation assay (See expt. 3.2.6). As mentioned before, the addition of rIL-2 to *in vitro* cultures, appears to discriminate against PCD versus AICD. IL-2 has been shown to protect naive T cells from PCD (Van Parijs *et al*, 1996) yet promote cell death

**FIGURE 6.8. Comparison of apoptosis in freshly isolated spleen cells from BALB/c mice infected with varying doses of mf by PI staining (A) and DNA fragmentation (B)**

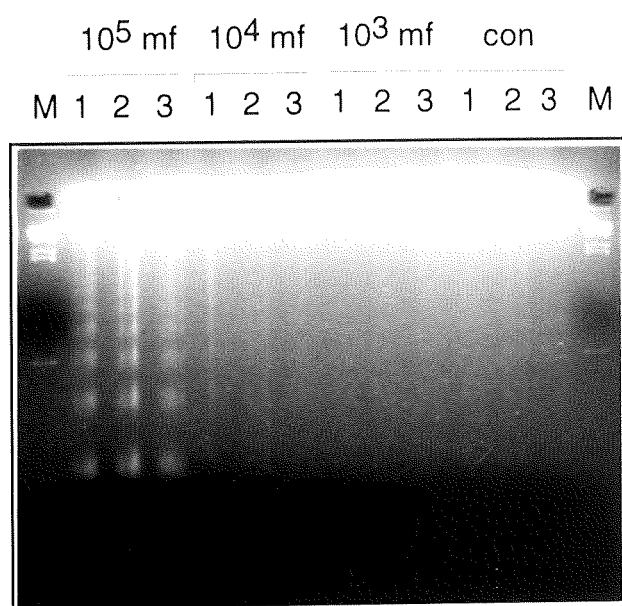
Groups of three BALB/c mice were injected s.c. with  $1 \times 10^5$ ,  $1 \times 10^4$  or  $1 \times 10^3$  mf or HBSS. At day 12 p.i., freshly isolated spleen cells were stained with PI or frozen before DNA extraction. (A) PI staining and flow cytometry was performed as described in Materials and Methods (Chapter 2). Data shown are the percentage of hypodiploid (apoptotic) cells in each sample  $\pm$ SD. \* Significant difference ( $p = 0.05$ ) between the percentage of hypodiploid cells in spleen cell cultures from mice infected with  $1 \times 10^5$  mf compared to the other groups. (B) Agarose gel electrophoresis was performed using DNA extracted from freshly isolated spleen cells. M is the molecular weight markers ( $\lambda$  HindIII). 1, 2 and 3 denotes the three mice per group.

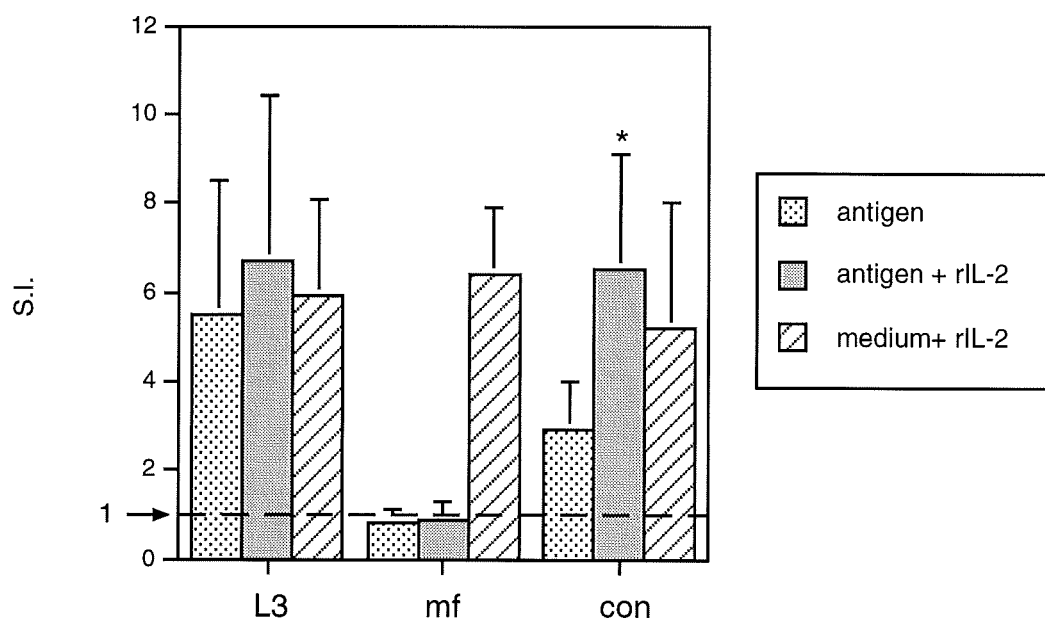
**Figure 6.8**

**A**



**B**





**FIGURE 6.9. The effect of exogenous IL-2 on antigen-driven proliferative response of spleen cells from BALB/c mice infected with  $1 \times 10^5$  mf**

The antigen-stimulated proliferation of spleen cells from BALB/c mice at day 12 p.i. with 50 L3,  $10^5$  mf or HBSS was measured by uptake of  $^3\text{H}$  thymidine after 96h of culture. The means of triplicate wells are expressed as stimulation indices (S.I.) = cpm with antigen/cpm with medium alone [dotted], cpm with antigen plus 50U/ml rIL-2/cpm with medium alone [solid grey], or cpm in medium plus 50U/ml rIL-2/cpm with medium alone [hatched]. The S.I.s represent the mean  $\pm$  SD of 5 animals per group. \* Significant difference ( $p < 0.05$ ) between value obtained with rIL-2 and with no treatment control.



by Fas-mediated AICD (Lenardo, 1991; Van Parijs *et al*, 1996). Therefore, the effect of IL-2 on the proliferative responses of spleen cells from mice infected with  $1 \times 10^5$  mf and L3 was analysed.

Groups of five mice were injected s.c. with  $1 \times 10^5$  mf, 50 L3 or HBSS (con) and at day 12 p.i. the mice were killed and spleen cell proliferation measured (Fig. 6.9). Cells were cultured for 96h with or without antigen, in the presence or absence of 50U/ml of rIL-2. The mean S.I. of spleen cells from mice infected with mf was  $<1$  ( $0.8 \pm 0.3$ ) (consistent with previous experiments, see 3.2.6). The addition of rIL-2 stimulated the proliferation of these cells when cultured in medium (S.I. =  $6.4 \pm 1.5$ ) but not antigen (S.I. =  $0.9 \pm 0.4$ ). In contrast, spleen cells from uninfected mice proliferated in response to IL-2 added to cells cultured in medium (S.I. =  $5.2 \pm 2.8$ ) or antigen (S.I. =  $6.5 \pm 2.6$ ). In addition, cells from L3-infected mice proliferated in response to both antigen alone (S.I. =  $5.5 \pm 3.0$ ) and IL-2 alone (S.I. =  $6.7 \pm 3.7$ ), although addition of rIL-2 to antigen-stimulated cultures did not further increase the S.I.

#### **6.2.6. Detection of apoptotic cells *in situ* in spleens from mice infected s.c. with mf**

Expts. 6.2.1, 6.2.2 and 6.2.4 demonstrated that a significant level of apoptosis could be detected in freshly isolated spleen cells specifically from mice infected with  $10^5$  mf. However, these cells had still been *ex vivo* for at least 2h at R.T. Therefore, it was important to determine whether apoptotic cells could be detected *in situ* in spleens from mf-infected mice. In this experiment, groups of three mice were injected s.c. with  $2 \times 10^5$  mf or HBSS. After 12 days, the mice were killed and the spleens sectioned and TUNEL stained using TdT enzyme and biotin-labelled dUTP. The inclusion of a streptavidin-peroxidase step in the labelling process acts to amplify the signal, permitting conventional histochemical identification of apoptotic cells (red-stained nuclei) by light micoroscopy.

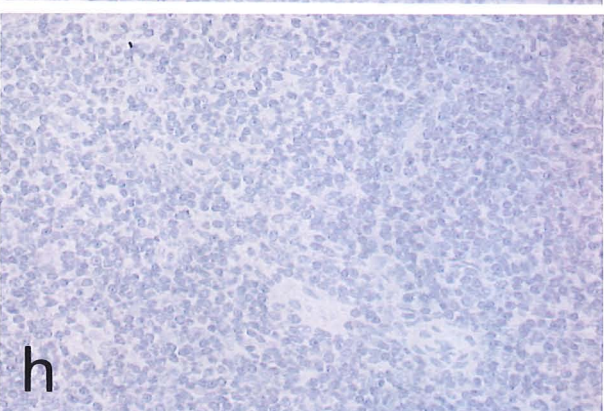
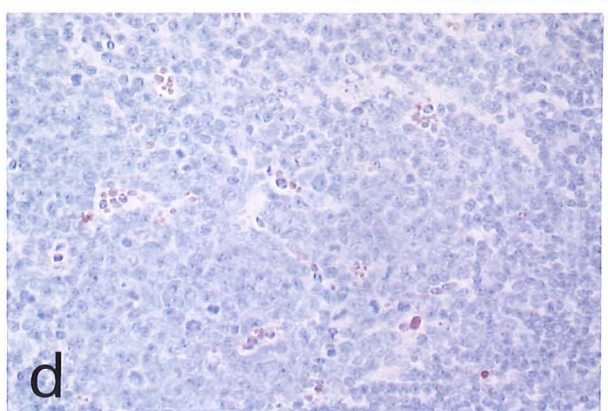
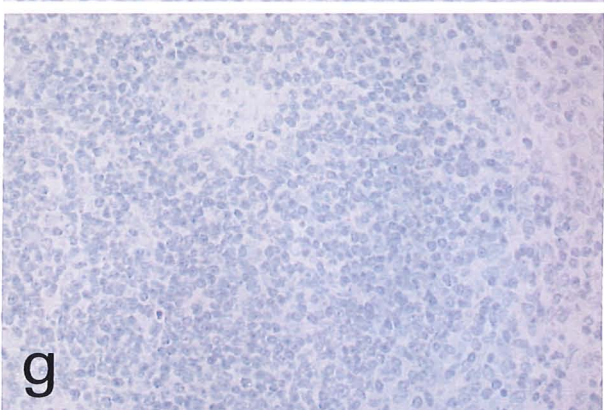
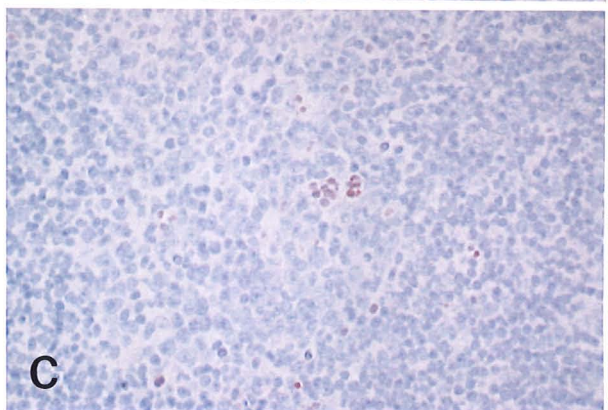
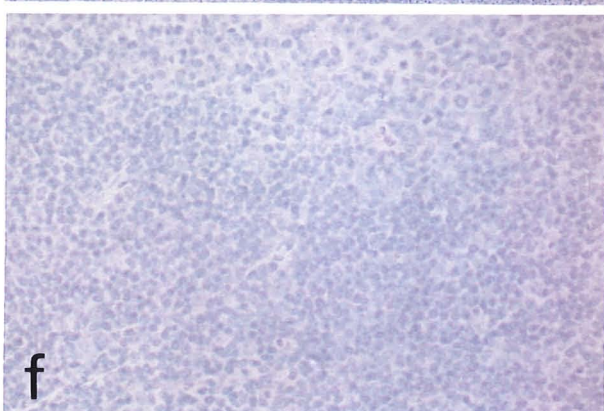
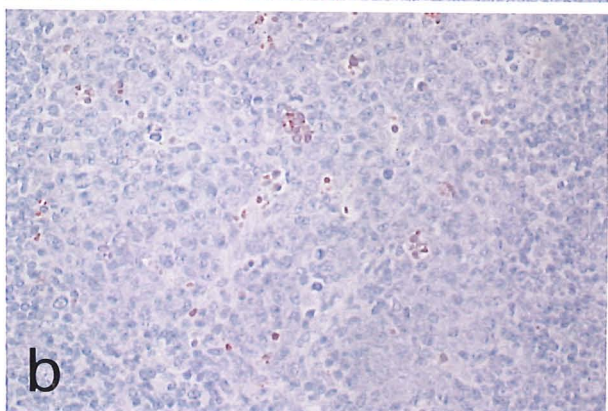
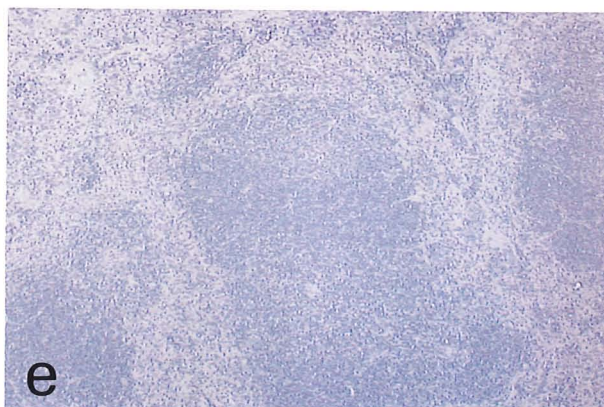
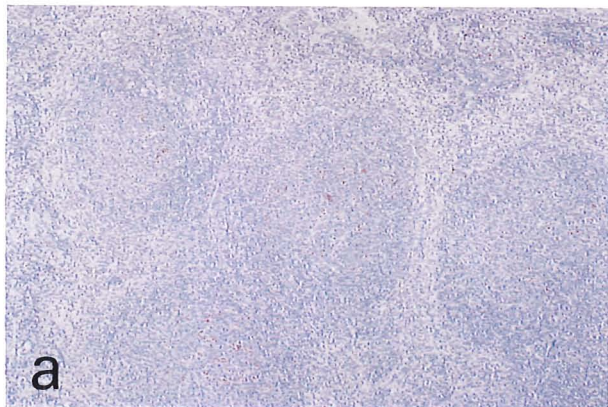
**FIGURE 6.10. *In situ* labelling of apoptotic cells in the spleens of BALB/c mice injected s.c. with  $2 \times 10^5$  mf or HBSS**

Groups of three mice were injected s.c. with  $2 \times 10^5$  mf (mf s.c.) or HBSS (con). At day 12 p.i., spleens were fixed in 4% buffered neutral formalin, embedded in paraffin wax and sectioned. Sections were TUNEL stained (See 2.4.5): (A) Test, nuclei containing nicked DNA indicating apoptotic cells. Panels a-d represent spleen sections from the three mice injected with mf. Panel a = x10 magnification of spleen section from mouse no. 1. Panels b, c and d = x40 magnification of spleen sections from mouse nos. 1, 2 and 3 respectively. Panel e-h represent spleen sections from the three mice injected with HBSS. Panel e = x10 magnification of spleen section from mouse no. 1. Panels f, g and h = x40 magnification of spleen sections from mouse nos. 1, 2 and 3 respectively. (B) Negative control, treated with all TUNEL reagents except dUTP/TdT, (C) Positive control, after pretreatment with DNaseI (20 $\mu$ g/ml).

**A**

mf s.c.

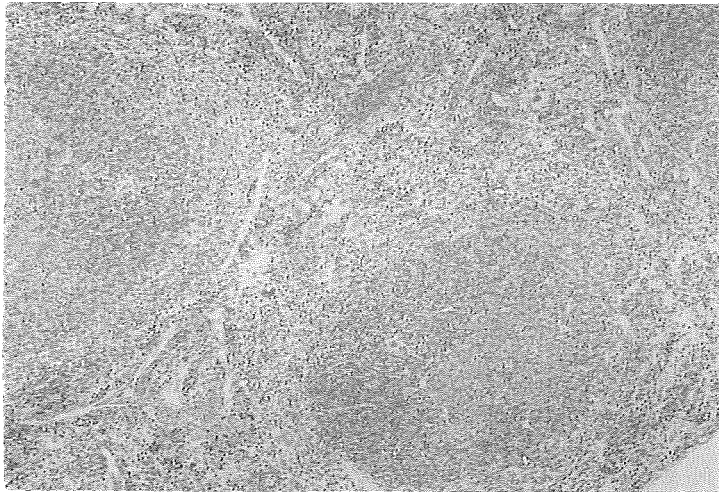
con



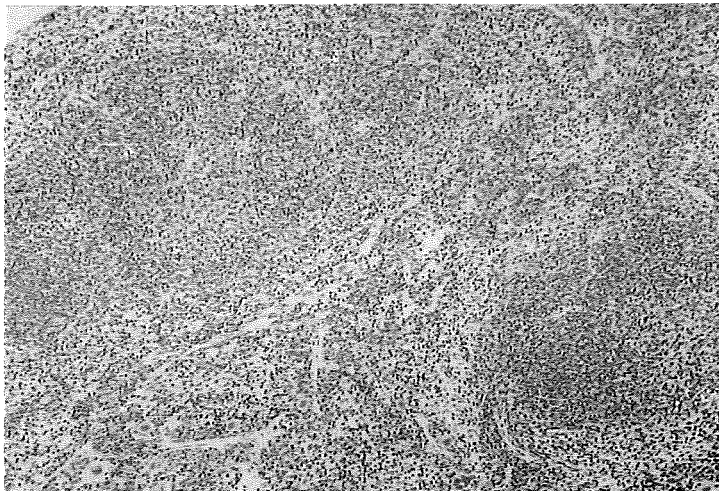


**Figure 6.10**

**B**



**C**



Two slides of each sections were prepared (See Materials and Methods, Chapter 2). One was labelled with all reagents (Test) and the other was treated identically except that the TdT enzyme/biotin-dUTP step was omitted (Negative controls). An additional slide from each group was pre-treated with DNase (Positive controls).

TUNEL clearly revealed a distinct pattern of staining in spleens from mf-infected mice (Fig 6.10A). Apoptotic cells were typically confined to clusters of cells in the white pulp area (lymphocyte containing area), apparently within secondary follicles. It was also possible to observe apoptotic cells being phagocytosed in these highly stained follicles. In contrast, the spleens of uninfected animals contained a few TUNEL-stained nuclei that are dispersed throughout the spleen. This pattern is normal for the spleens of uninfected mice (Gavrieli *et al*, 1992). No red brown staining was observed in any negative control slides (Fig 6.10B). Pre-treatment with DNase I caused an intensive staining of nuclei in all sections, indicating that the nuclei in sections from each group are equally accessible to the TUNEL labelling reaction (Fig 6.10C).

#### **6.2.7. Detection of apoptotic cell death in spleens of lpr/lpr mice infected s.c. with mf**

In this experiment, MRL-lpr/lpr homozygous (Fas deficient) and MRL-lpr/C57BL/6 heterozygotes (Fas positive) were used to assess the role of Fas/FasL interaction in the induction of apoptosis following infection with mf. Groups of five lpr/lpr mice and four lpr/C57 mice were injected s.c. with  $1 \times 10^5$  mf or HBSS (con). Freshly isolated spleen cells were stained with PI or frozen before DNA extraction. The proliferative response of the remaining spleen cells to antigen and ConA was also assessed.

#### **PI staining/FACS analysis and DNA fragmentation**

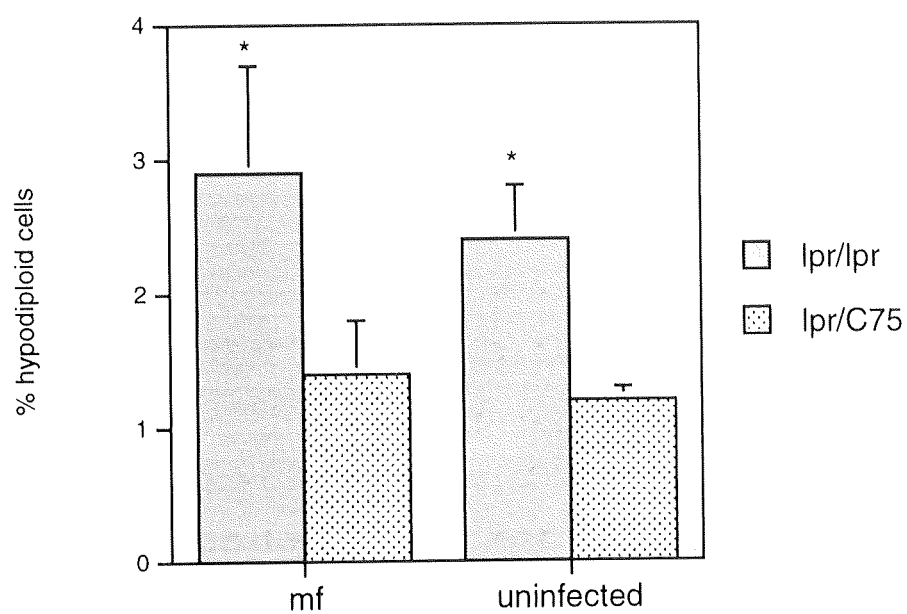
Fig 6.11A shows that infection with mf did not result in an increase in the level of hypodiploid cells in the spleens of lpr/lpr mice (mf= $2.9 \pm 0.8\%$  compared to con= $2.4 \pm 0.4\%$ ,  $p > 0.05$ ). However, the percentage of hypodiploid cells in spleen cell

**FIGURE 6.11. Comparison of apoptosis in freshly isolated spleen cells from lpr/lpr and lpr/C57 mice following infection with  $1 \times 10^5$  mf by PI staining (A) and DNA fragmentation (B)**

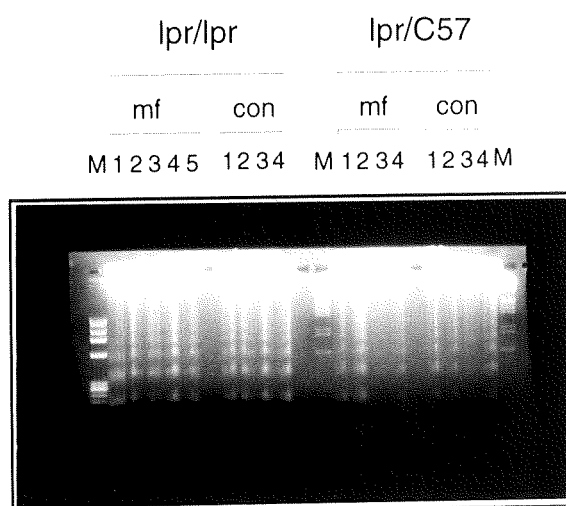
Groups of five lpr/lpr (Fas deficient) and four lpr/C57 (Fas expressing) mice were injected with  $1 \times 10^5$  mf or HBSS. At day 12 p.i., freshly isolated spleen cells were stained with PI or frozen before DNA extraction. (A) PI staining and flow cytometry performed as described in 2.4.3. Data shown are the percentage of hypodiploid (apoptotic) cells in each sample  $\pm$ SD. (B) Agarose gel electrophoresis was performed using DNA extracted from freshly isolated spleen cells. M is the molecular weight markers ( $\lambda$  Hind III). The numbers denote the individual mice in each group.

Figure 6.11

A



B



preparations from lpr/C57 mice infected with  $10^5$  mf ( $1.4 \pm 0.4\%$ ) was also not significantly higher than that of spleen cells from uninfected lpr/C57 mice ( $1.2 \pm 0.1\%$ ,  $p > 0.05$ ). In fact, the levels of hypodiploid cells in spleens from both mf-infected lpr/lpr and lpr/C57 mice were lower than that observed previously in the majority of experiments with BALB/c mice that had been infected with the same number of mf (Expts. 6.2.1 and 6.2.3). However, the proportion of hypodiploid cells was significantly higher in spleen cell preparations from both mf-infected and uninfected lpr/lpr compared to both groups of lpr/C57 mice.

Despite the low level of apoptotic cells, assessed by PI staining, DNA fragmentation patterns were observed in freshly isolated spleen cells from all four groups (Fig 6.11B).

### **Proliferation Assays**

The *in vitro* proliferative and cytokine responses of spleen cells from infected and uninfected lpr/lpr and lpr/C57 mice to antigen and ConA were assessed since this was the first time that these mutant mouse strains had been used for infection with *B. pahangi*. The proliferative and cytokine responses of spleen cells from C57BL/6 mice have already been shown to be almost identical to those of BALB/c mice (See expt. 5.2.6).

In this experiment, the ConA-stimulated proliferative response of spleen cells from both groups of lpr/lpr mice was impaired in comparison to cells from lpr/C57 mice (Table 6.1). However, the ConA-stimulated proliferative response of lpr/lpr and lpr/C57 mice was substantially lower than that obtained previously with spleen cells from BALB/c mice (See expt. 3.2.1). However, spleen cells from lpr/lpr and lpr/C57 mice infected with  $1 \times 10^5$  mf were unable to proliferate in response to antigen after 3d in culture and displayed S.I. $<1$  after 4d. Neither uninfected lpr/lpr or lpr/C57 mice proliferated in response to antigen after 3d nor exhibited S.I. $<1$  after 4d.



**TABLE 6.1. Proliferative responses of spleen cells from MRL-lpr/lpr and MRL-lpr/C57BL/6 mice<sup>a</sup> infected with  $1 \times 10^5$  mf to ConA and *B. pahangi* antigen.**

	proliferation (S.I.) <sup>b</sup>			
	lpr/lpr		lpr+/-	
stimulus	mf	uninfected	mf	uninfected
ConA 48h	$17.2 \pm 7.9$	$17.8 \pm 5.0$	$49.3 \pm 22.3$	$55.3 \pm 30.2$
Ag 3d	$1.4 \pm 0.5$	$1.5 \pm 0.1$	$1.1 \pm 0.3$	$1.4 \pm 0.5$
Ag 4d	$0.9 \pm 0.2$	$1.7 \pm 1.2$	$0.3 \pm 0.2$	$2.9 \pm 3.8$

<sup>a</sup> Mice were injected as described in legend to Fig. 6.13.

<sup>b</sup> At day 12 p.i. proliferation of spleen cells at  $5 \times 10^6$  cells/ml to (A)  $1 \mu\text{g/ml}$  of ConA or (B)  $10 \mu\text{g/ml}$  of antigen was measured by uptake of  $^3\text{H}$  thymidine after 48h (ConA), 72h and 96h (antigen) of culture. The means of triplicate wells are expressed as stimulation indices (S.I.) = cpm with ConA or Ag/cpm with medium alone. The values are means  $\pm$  SD of five MRL-lpr/lpr and four MRL-lpr/C57BL/6 mice per group. \* Significant difference ( $p < 0.05$ ) compared to uninfected controls.

**TABLE 6.2. Cytokine responses of spleen cells from MRL-lpr/lpr and MRL-lpr/C57BL/6 mice<sup>a</sup> infected with  $1 \times 10^5$  mf to ConA and antigen.**

	cytokine levels (U/ml) <sup>b</sup>				
	lpr/lpr		lpr/C57		
stimulus	mf	uninfected	mf	uninfected	
Ag	$2.2 \pm 0.3^*$	0	0	0	<b>IL-2</b>
ConA	0	0	$28.8 \pm 20.7$	$70.7 \pm 124.7$	
ConA	$77.0 \pm 49.6$	$77.9 \pm 39.1$	$84.2 \pm 7.2$	$81.1 \pm 20.2$	<b>IFN-<math>\gamma</math></b>
ConA	$0.44 \pm 0.09$	$0.46 \pm 0.06$	$0.29 \pm 0.06$	$0.22 \pm 0.08$	<b>IL-10</b>

<sup>a</sup> Mice were injected as described in legend to Fig. 6.13.

<sup>b</sup> At day 12 p.i. cytokine levels in 48h supernatants from spleen cells at  $1 \times 10^7$  cells/ml in response to ConA ( $5 \mu\text{g/ml}$ ) or antigen ( $10 \mu\text{g/ml}$ ) were measured by 2-site ELISA with reference to standards of known concentration (U/ml). A value of 0 indicates a reading below the sensitivity of the assay. The values are means  $\pm$  SD of five lpr/lpr and four lpr/C57 mice per group. Assay sensitivities were 1.95 U/ml (IL-2), 15.6 U/ml (IFN- $\gamma$ ), 0.195 U/ml (IL-10), 0.781 U/ml (IL-4) and 0.391 U/ml (IL-5). \* Significant difference ( $p < 0.05$ ) compared to uninfected controls.

Consistent with the poor ConA-induced proliferation, spleen cells from all groups of mice produced significantly less IL-2 to ConA (Table 6.2) than that previously detected in spleen cell cultures from BALB/c mice. In fact, no ConA-stimulated IL-2 was detected in any of spleen cell cultures from lpr/lpr mice and two out of four of the cultures from lpr/C57 mice, despite a very low assay sensitivity ( $\geq 1.92$  U/ml). However, all spleen cell cultures from mf-infected lpr/lpr mice contained a low level of antigen-specific IL-2. Surprisingly, a moderate level of ConA-stimulated IFN- $\gamma$  and a low, but significant, level of ConA-stimulated IL-10 was produced by spleen cells from all groups of mice. However, no antigen or ConA-driven IL-4 or IL-5 could be detected in any of the cultures.

#### **6.2.8. Detection of apoptotic cell death *in situ* in spleens of lpr/lpr mice infected s.c. with mf**

In contrast to their relative resistance to apoptosis *in vivo* and AICD *in vitro*, T cells from MRL lpr/lpr mice are known to undergo spontaneous cell death when removed from the animal at a much accelerated rate compared to wild type mice (Van Houten and Budd, 1992). Although the reason for this is not clear, it may be due to background genes in the MRL strain, since a similar phenomenon is also observed in MRL lpr/C57 heterozygotes. This may explain why freshly isolated spleen cells from mf-infected and uninfected MRL lpr/lpr and MRL lpr/C57 mice contain significant DNA fragmentation and display such poor *in vitro* proliferative and cytokine responses to ConA and parasite antigen (Expt 6.7). Therefore, it was then investigated whether an increased number of apoptotic cells could be detected *in situ* in the spleen of mf-infected lpr/lpr mice compared to uninfected lpr/lpr controls. Spleens from three lpr/lpr mice injected s.c. with  $1 \times 10^5$  mf and two controls (HBSS) were removed at day 12 p.i., fixed and sectioned for TUNEL, as described in Expt 6.7.

In contrast to the results of Expt 6.2.7, using mf-infected and uninfected BALB/c mice, no difference in the pattern of TUNEL staining could be observed between the spleen

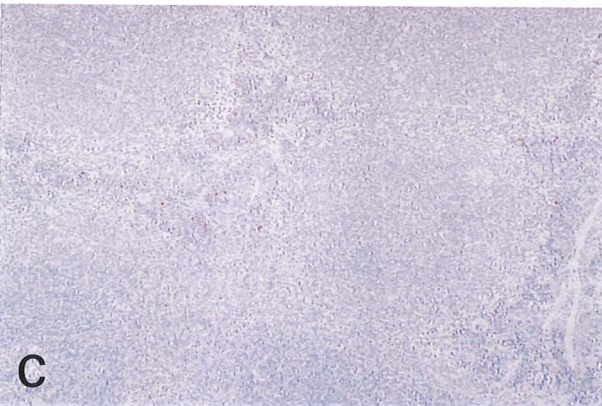
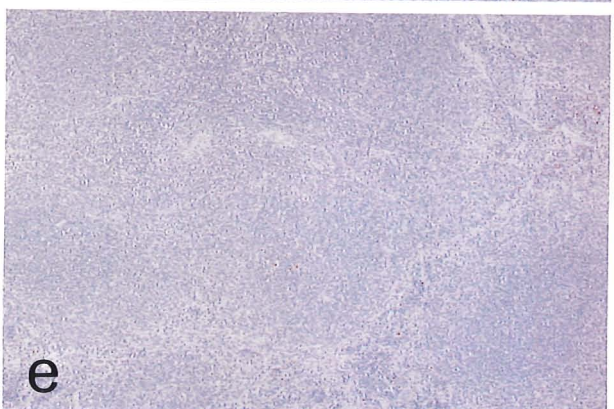
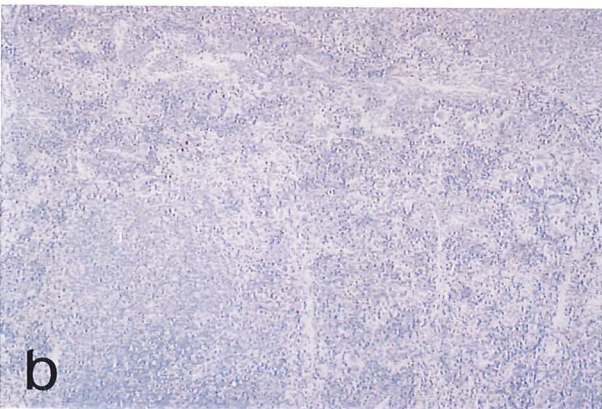
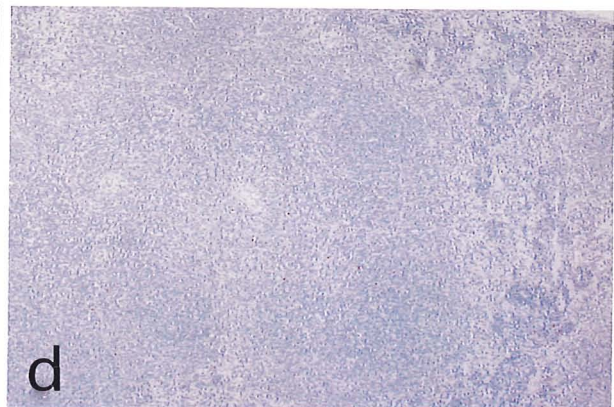
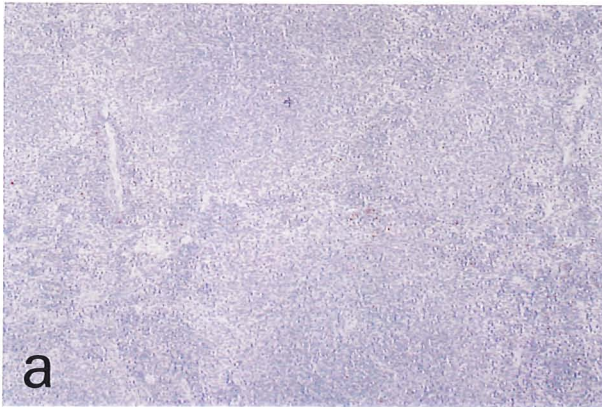
**FIGURE 6.12. *In situ* labelling of apoptotic cells in the spleens of lpr/lpr mice injected s.c. with  $1 \times 10^5$  mf or HBSS**

Groups of three lpr/lpr mice were injected with  $1 \times 10^5$  mf or two control mice (HBSS). At day 12 p.i. spleens were removed and treated as described in the legend to Fig. 6.10.. Sections were TUNEL stained (see 2.4.5): (A) Test, nuclei containing nicked DNA indicate apoptotic cells. Panels a-c represent x10 magnification of spleen sections from the three mice injected with mf. Panels d and e represent x10 magnification of spleen sections from the two mice injected with HBSS. (B) Negative control, treated with all TUNEL reagents except dUTP/TdT. (C) Positive control, after pretreatment with DNaseI (20 $\mu$ g/ml).

**A**

lpr/lpr mf s.c.

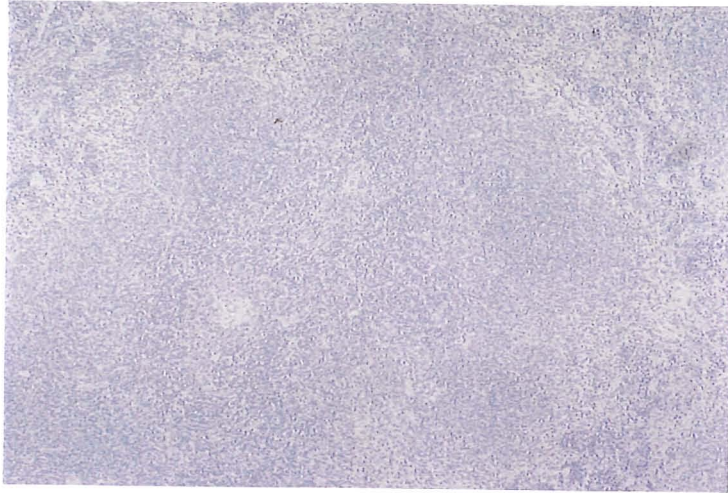
lpr/lpr con



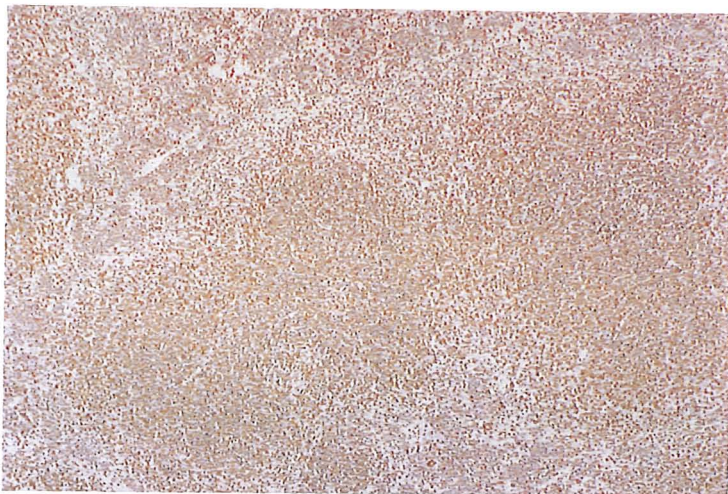


**Figure 6.12**

**B**



**C**



of mf-infected and uninfected lpr/lpr mice (Fig. 6.12A). A diffuse, low level staining for apoptotic cells was observed throughout the sections from both groups of mice. No red-brown staining was observed in any of the negative control slides (Fig. 6.12B). In sections from both groups of lpr/lpr mice virtually all nuclei have stained red following pre-treatment with DNase I indicating that there is no difference in efficiency of the TUNEL labelling reaction between the two groups (Fig 6.12C).

#### **6.2.9. Comparison of apoptotic cell death *in situ* in spleens of BALB/c mice infected s.c. versus i.v. with mf versus L3**

In view of the enhanced sensitivity of the TUNEL technique compared to the other methods for detecting apoptotic cells, it was important to ensure that mice infected with L3, that do not possess apoptotic spleen cells in freshly isolated samples (Expt. 6.2.1), do not display the clustered pattern of apoptotic nuclei in secondary follicles as observed in the spleens of mice infected s.c. with mf (Expt. 6.2.6). In addition, it was interesting to investigate whether mf, in their natural environment (the bloodstream), were able to induce a similar pattern of TUNEL stained cells in the spleen compared to animals infected s.c. with mf. In this experiment, groups of three mice were injected s.c. with  $2 \times 10^5$  mf, 50 L3 or HBSS or i.v. (tail vein) with  $2 \times 10^5$  mf. At day 12 p.i., spleens were removed and processed before TUNEL staining as described in Expt 6.2.6.

Consistent with the results of Expt 6.2.6, a distinctive pattern of TUNEL staining, i.e. clusters of significant numbers (average  $\approx 20$ ) of apoptotic cells in secondary follicles, were observed in spleens from mice infected s.c. with mf but not in the spleens of uninfected controls (Fig 6.13A). In contrast, the pattern of TUNEL-staining in spleens of mice infected with L3 resembled that of uninfected controls. Spleens from both L3-infected and uninfected mice contained a few apoptotic cells that were scattered throughout the sections. TUNEL-stained spleen sections of mice infected i.v. with mf, on the other hand, were almost identical to those of mice infected s.c. with mf; the majority of follicles in sections from both groups of mice contained intensely staining

**FIGURE 6.13. *In situ* labelling of apoptotic cells in the spleens of BALB/c mice injected s.c. with  $2 \times 10^5$  mf, 50 L3 or HBSS or i.v. with  $2 \times 10^5$  mf**

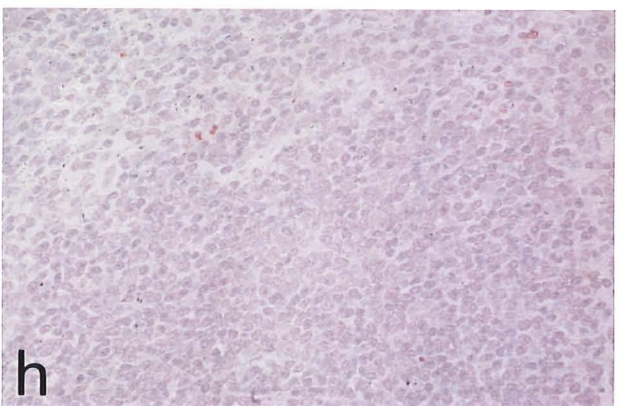
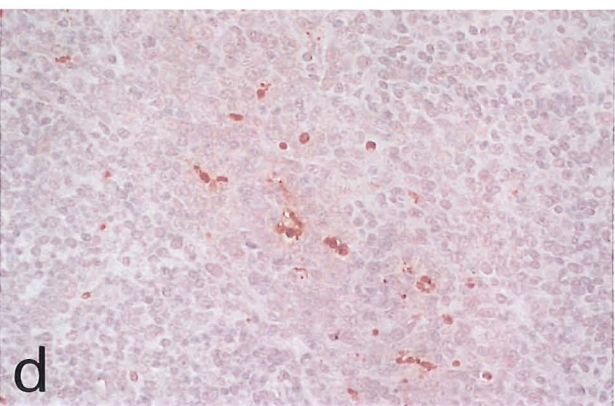
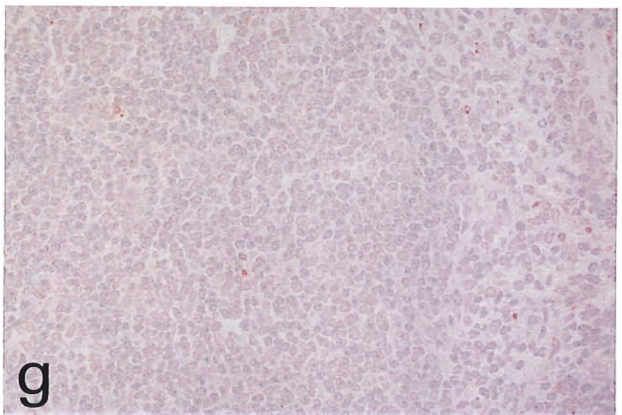
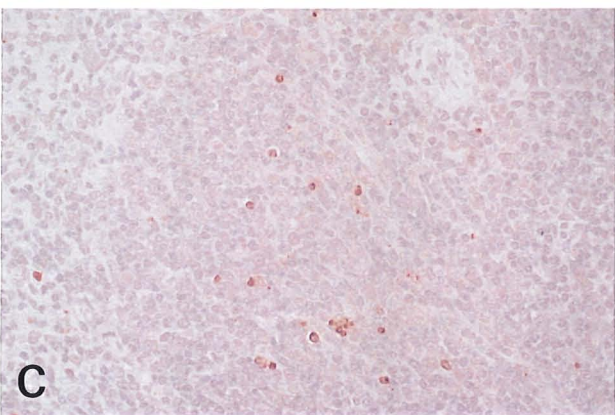
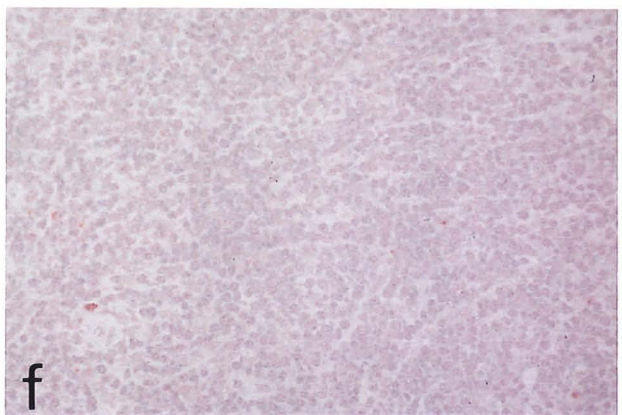
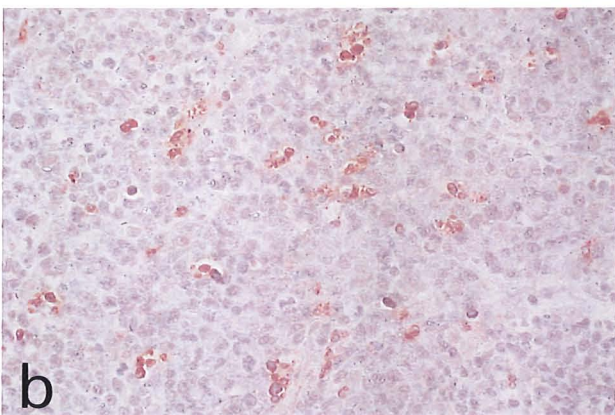
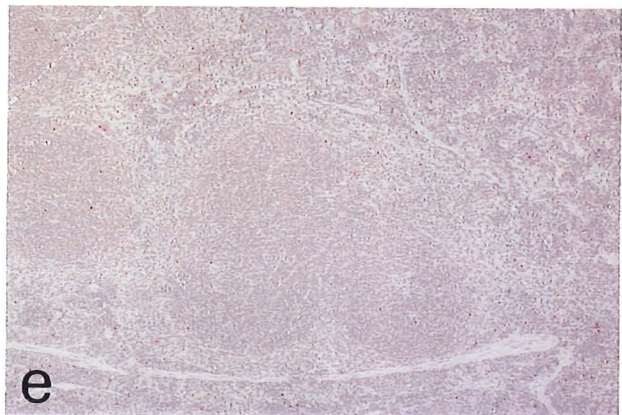
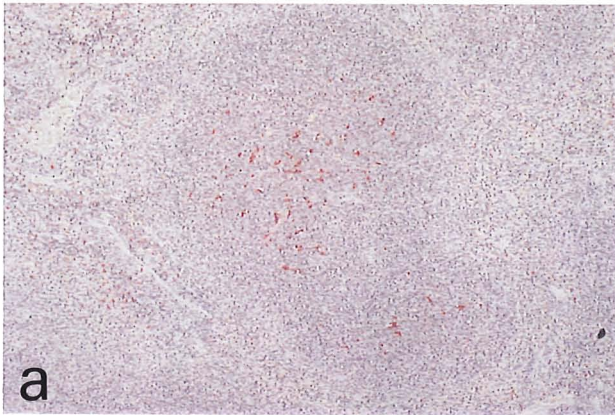
Groups of three mice were injected s.c. with  $2 \times 10^5$  mf, 50 L3 or HBSS or i.v. with  $2 \times 10^5$  mf. At day 12 p.i. spleens were removed and treated as described in the legend to Fig. 6.10. (A) Test, nuclei containing nicked DNA indicating apoptotic cells. Panels a-d represent spleen sections from the three mice injected s.c. with mf (mf s.c.). Panel e-h represent spleen sections from the three mice injected s.c. with L3 (L3 s.c.). Panels i-l represent spleen sections from the three mice injected i.v. with mf (mf i.v.). Panels m-p represent spleen sections from the three mice injected s.c. with HBSS (con). Panels a, e, i, and m = x10 magnification of the spleen section from mouse no. 1 in each group. Panels b, f, j, and n = x40 magnification of the spleen section from mouse no. 1 in each group. Panels c, g, k and o = x40 magnification of the spleen section from mouse no. 2 in each group. Panels d, h, l and p = x40 magnification of the spleen section from mouse no. 3 in each group. (B) Negative control, treated with all TUNEL reagents except dUTP/TdT. (C) Positive control, after pretreatment with DNaseI (20 $\mu$ g/ml).



**A**

mf s.c.

L3 s.c.

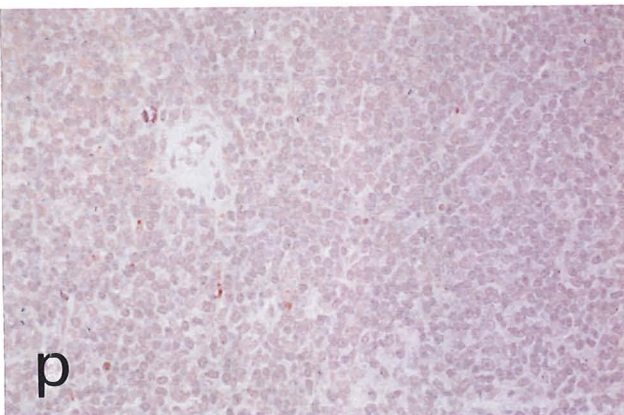
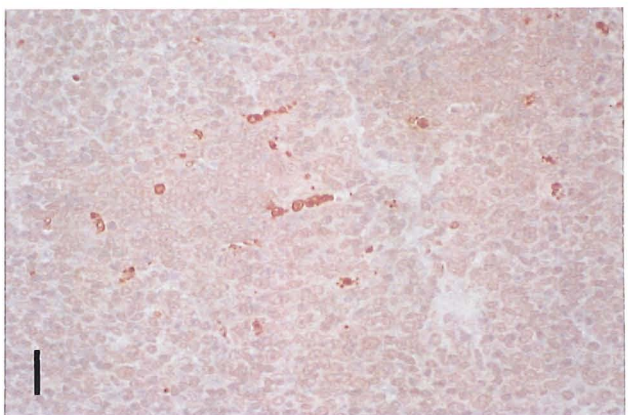
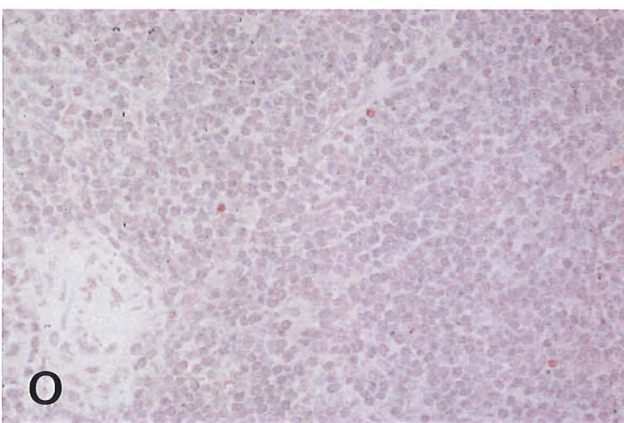
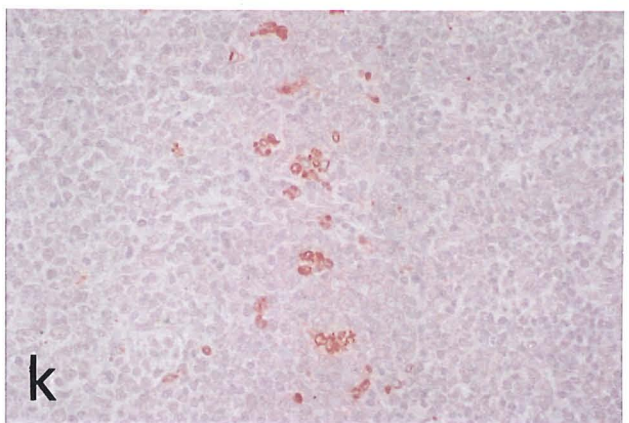
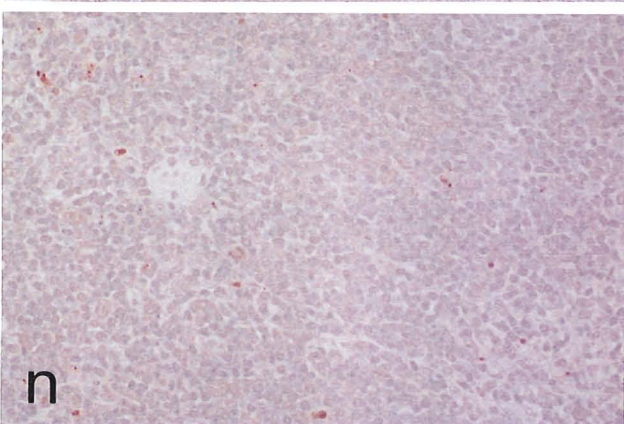
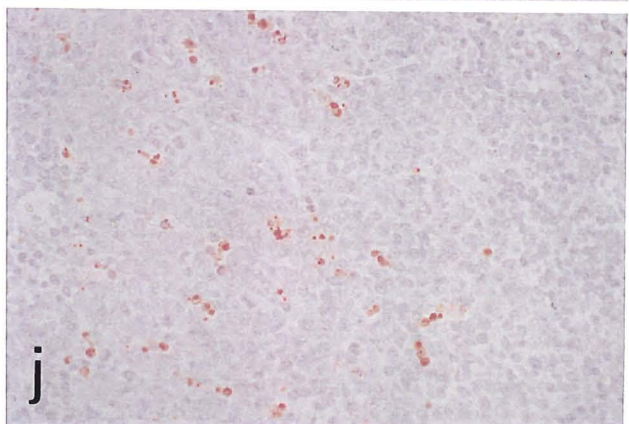
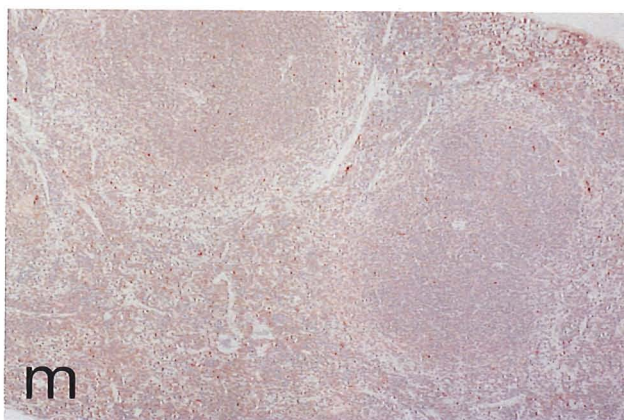
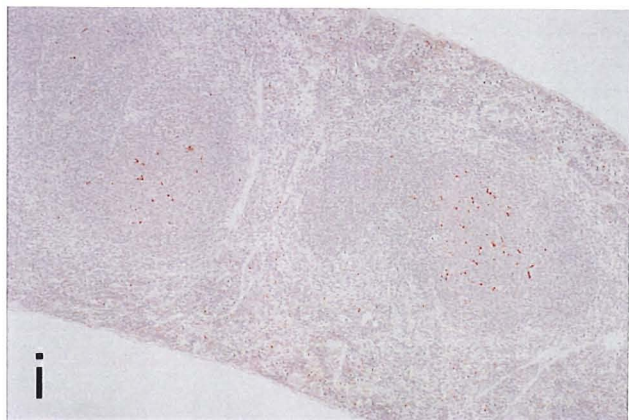




**A**

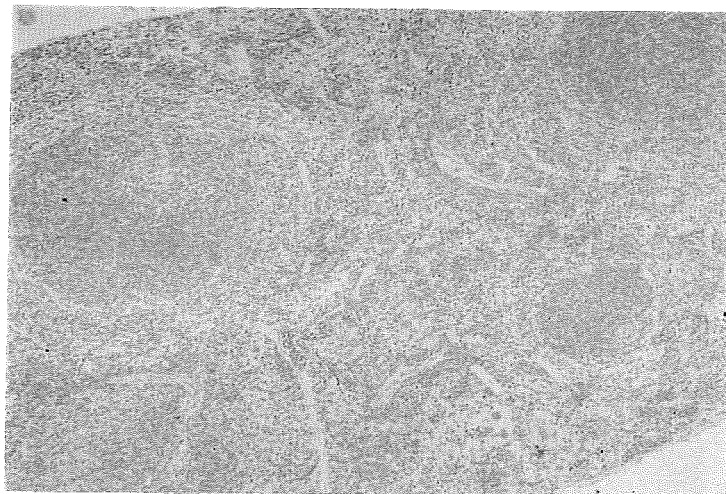
mf i.v.

con

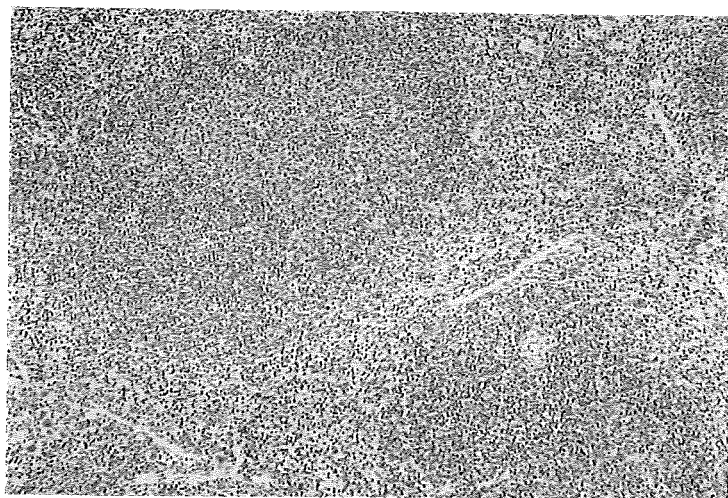


**Figure 6.13**

**B**



**C**



clusters of apoptotic cells. However, one out of three of the mice in the i.v. injected group possessed only 3 such follicles (mouse no. 3), suggesting that this mouse had not received the full dose of mf. No red brown staining was observed in any negative control slides (Fig 6.13*B*). Pre-treatment with DNase I causes an intensive staining of nuclei in all sections, indicating that the nuclei in sections from each group are equally accessible to the TUNEL labelling reaction (Fig. 6.13*C*).



### 6.3. Discussion

The results presented in this chapter describe the induction of apoptotic death in the spleens of BALB/c mice infected with mf. Detection of apoptotic cells *in situ* and in freshly isolated cells was exclusive to infection with mf and may explain the inability of spleen cells from these mice to proliferate in response to antigen *in vitro*.

Freshly isolated spleen cells from mf-infected mice contained apoptotic (hypodiploid) cells, as assessed by PI staining, and fragmented DNA. In contrast, DNA prepared from freshly isolated spleen cells from L3-infected and uninfected mice was intact and there was no increase in the number of hypodiploid cells. Consistent with these results, spleen sections from mice infected s.c. with mf, but not L3 or HBSS, exhibit a distinctive pattern of TUNEL-stained clusters of cells within secondary follicles. It was also possible to observe apoptotic bodies and apoptotic cells being phagocytosed within these positive follicles. Furthermore, spleen sections from mice injected i.v. with mf contained an almost identical pattern of TUNEL-stained cell clusters within many of the follicles to that observed in spleens following s.c. infection. TUNEL-stained spleen sections from L3-infected and uninfected controls were very similar; a few individual apoptotic cells were scattered throughout sections from both sets of mice. Furthermore, the induction of apoptosis following infection with mf appears to be dose dependent since DNA fragmentation and cells with hypodiploid DNA content were detected in freshly isolated spleen cells from mice infected s.c. with  $1 \times 10^5$ , but not  $1 \times 10^4$  or  $1 \times 10^3$  mf. In addition, freshly isolated spleen cells from two out of three mice infected with  $1 \times 10^4$  mf displayed barely discernible DNA fragmentation suggesting that a lower level of apoptosis may be occurring in animals injected with less than  $1 \times 10^5$  mf that was below the sensitivity of the PI staining and DNA fragmentation assays. Since the FACS analysis program gated only on PI stained lymphocytes and it is the T cell proliferative response that is defective in mf-infected mice, the assumption is that it is T cells that are undergoing apoptosis. To confirm directly that T cells are the predominant

cell type undergoing apoptosis in mf-infected mice, PI staining could be combined with conventional fluorescent surface labelling using a T cell marker. Alternatively, TUNEL-stained spleen sections could be co-stained for T cell areas.

The results of experiments analysing cultured spleen cells were not as clear-cut because of the high background levels of hypodiploid cells and DNA fragmentation in spleen cell cultures from all groups of mice, including uninfected controls. This background was due to spontaneous cell death in spleen cell cultures, reported previously (Razvi and Welsh, 1993), since it could be blocked if the cells were incubated over-night at 4°C rather than 37°C. However, significant differences could still be detected between cultures of spleen cells from infected compared to uninfected mice. At 120h the % of apoptotic cells was elevated in antigen-stimulated spleen cell cultures from L3- and mf-infected mice compared to the level in antigen-stimulated cultures from uninfected controls. In contrast, no difference was detected in the % apoptotic cells at 120h from the three groups of mice when incubated without antigen.

Furthermore, it is important to note that the pattern of % viability and % specific (antigen-stimulated) cell death in normal cultures over time, revealed by trypan blue staining, correlated well with the % apoptotic cells, assessed by PI staining and FACS analysis. The positive association between the % of apoptotic cells and the presence of antigen in 120h cultures of spleen cells from L3- and mf-infected, but not uninfected mice was consistent with a significant level of specific cell death in cultures from L3- and mf-infected, but not uninfected mice at the same time point. In addition, at 96h the % of apoptotic cells in antigen-stimulated cultures of spleen cells from L3-infected, but not mf-infected, mice was elevated compared to the same cells cultured in medium alone. This correlated with a significant level of specific cell death in the 96h cultures of spleen cells from L3-infected, but not mf-infected mice.

However, it was not clear why spleen cells from L3-infected mice displayed a similar pattern of cell death in culture as cells from mf-infected mice, yet are known to still proliferate in response to antigen at 112h (See 3.2.6). Consistent with an increased % of apoptotic cells, antigen-stimulated spleen cell cultures from L3 and mf-infected mice contained a reduced % of viable cells after 120h, compared to spleen cells from uninfected mice. However, on closer examination it was apparent that the % of viable cells in antigen-stimulated cultures from mf-infected mice was in fact significantly lower than in cultures from L3-infected mice. Despite this observation, the % specific cell death at 120h, that accounts for variation in cell number in culture due to differences in proliferation, is identical in cultures from L3- and mf-infected mice. These findings suggest that the increased cell death in cultures of spleen cells from L3-infected mice at 96 and 120h compared to uninfected controls, as a result perhaps of partial- or over-activation, may be obscured in proliferation assays by the generation of new cells ( $S.I. > 1$  and significantly higher than S.I. of spleen cells from uninfected controls at 88 and 112h). In contrast, in the absence of cell division in antigen-"stimulated" cultures from mf-infected mice, the augmented cell death at 120h compared to cultures from L3-infected and uninfected mice would be discernable in proliferation assays ( $S.I. < 1$  at 112h).

After this initial characterisation, attempts were made to determine the possible mechanism underlying the T cell apoptosis detected in freshly isolated spleen cells following infection with mf. The first and obvious strategy was to categorise it as PCD or AICD. The correlation between the reduction in cell viability and increase in number of apoptotic cells after 120h of culture in the presence of antigen (but not medium alone) and  $S.I. < 1$  after 112h in antigen proliferation assays suggests that AICD is the mechanism. A scenario could be envisaged in which antigen-specific spleen cells are sensitised, following infection with mf, to undergo apoptosis *in vivo* (TUNEL positive) and that the increased cell death being observed in the antigen-stimulated (but not

medium only) cultures, by PI and trypan blue staining, is simply a continuation of this process *in vitro*.

Exogenous IL-2 has been shown to augment AICD of normal T cells and T cell hybridomas (Van Parijs *et al*, 1996) and to block PCD of naive T cells (Lenardo, 1991; Van Parijs *et al*, 1996). However, the measurement of proliferation, after the inclusion of rIL-2 in 112h spleen cell cultures of mf-infected mice, did little to differentiate between AICD and PCD. Spleen cells from mf-infected mice, that exhibited a S.I.<1 in the presence of antigen, proliferated vigorously when cultured in medium supplemented with IL-2. However, in the presence of antigen, the addition of IL-2 did not stimulate the proliferation of spleen cells from mf-infected mice nor did it cause the S.I. to decrease further. In contrast, cultures of cells from uninfected mice proliferated in response to IL-2 in the presence or absence of antigen.

Therefore, the effect of IL-2 is difficult to interpret in terms of the possible mechanism of apoptosis. In the absence of antigen, is IL-2 capable of stimulating the proliferation of antigen-primed T cells or does IL-2 only stimulate the proliferation of other T cells in the cultures? However, the presence of antigen is clearly preventing normal IL-2 responsiveness, suggesting that antigen-reactive cells from mf-infected mice are suppressing the activity of other T cells in the antigen-stimulated cultures. However, with proliferation as the only assay read-out, it was not possible to assess whether IL-2 increased the level of apoptosis in these antigen-stimulated cultures, although the fact that the S.I. did not decrease further suggests not. Despite the inevitable high background level of apoptosis in spleen cell cultures, PI staining and FACS analysis of IL-2-treated cultures may still give a more sensitive and accurate assessment of the incidence of apoptosis. However, it is important to realise that the role of IL-2 in augmenting AICD may not be as absolute as first suggested, since IL-2 protects against apoptosis of activated (CD54RO<sup>+</sup>) CD4<sup>+</sup> T cells from virally infected humans that were cultured in medium alone (Akbar *et al*, 1993) and CD4<sup>+</sup> T cells from HIV-



infected asymptomatic individuals stimulated in culture with PWM or SAg (Groux *et al*, 1992).

In other infection-related examples of apoptosis, AICD appears to be the underlying mechanism since spleen cells from mice infected with either *T. cruzi* (Lopes *et al*, 1995; Lopes and DosReis, 1996) or LCMV (Razvi and Welsh, 1993), but not uninfected mice, display severely impaired proliferation and undergo rapid apoptosis in culture following stimulation with ConA or via the TCR. In addition, pre-treatment of spleen cells from LCMV-infected mice with IL-2, accelerates apoptosis upon subsequent stimulation with anti-CD3 or ConA. In contrast, although antigen-specific proliferation was absent in spleen cells cultures from mf-infected mice, their ConA-driven proliferation was normal (3.2.6). Furthermore, the accelerated decrease in viability and increased number of apoptotic cells occurred only in spleen cell cultures from mf-infected mice in the presence of antigen. However, the absence of generalised immunodeficiency in mf-infected mice due to T cell apoptosis, compared to acute viral and protozoal infections, may simply reflect a lower number of antigen-reactive T cells and, of course, the absence of pathogen infected T cells or APCs in mf-infected animals. An exact mechanism for T cell apoptosis has yet to be described that would directly account for immunosuppression and parasite persistence in hosts infected with intracellular pathogens.

The findings so far do not discount a mechanism of PCD underlying mf-related apoptosis. Following infection with mf, apoptosis of antigen-specific T cells may occur *in vivo* due to insufficient costimulation at the time of antigen presentation. Induction of apoptosis and anergy are thought to be closely related since they can both occur following non-optimal T cell stimulation; functional activation results in proliferation and cytokine production (Strasser, 1995a). T cell anergy and T cell death by apoptosis are both thought to contribute to tolerance (defined as the absence of *in vitro* proliferative response to antigen) induced by injection of antigen in the absence of an adjuvant

(Kearney *et al*, 1994). Certainly cells from mf-infected mice display evidence of anergy *in vitro*, in that they produce cytokines (IFN- $\gamma$ ) but do not proliferate (Evavold and Allen, 1991). Furthermore, apoptosis in the spleen is evident following infection with mf by the s.c. (PI staining, DNA fragmentation, TUNEL) and i.v. routes (TUNEL). S.C. infection is not the natural route; it is not known where the mf go following injection and antigen presentation to reactive T cells may be inefficiently costimulated. In addition, mf in their natural environment of the blood circulation have direct access to the spleen, where mf-derived antigens could be presented by resting B cells and unactivated macrophages. Soluble protein antigens are thought to induce T cell unresponsiveness when injected i.v. because they are processed and presented by APC in the spleen without inducing their costimulatory activity (Johnson and Jenkins, 1994).

In a model of oral tolerance, the level of lymphocyte death in cultures from mice fed with OVA could not be accounted for by death of OVA-specific T cell clones alone (Garside *et al*, 1996). Therefore, factors secreted by tolerized cells *in vitro* may suppress and ultimately cause the death of any antigen (i.e. non just OVA)-driven cells in the vicinity. Since tolerised cells produced high levels of TGF- $\beta$ , it was suggested that this cytokine may be responsible for mediating the bystander suppression/killing. This situation resembles that of spleen cell cultures from mf-infected mice that lose their IL-2 responsiveness when cultured in the presence of antigen. However, although TGF- $\beta$  is known to be a potent inhibitor of T cell proliferation (Dasch *et al*, 1989) there is no direct evidence that it can induce T cell apoptosis. Furthermore, in contrast to the situation in spleen cultures from mf-infected mice, the apoptosis of orally tolerised cells *in vitro* is prevented by the addition of antigen (Garside *et al*, 1996).

Furthermore, IFN- $\gamma$  is a more obvious candidate than TGF- $\beta$ , in mediating T cell apoptosis in mf-infected mice, since it is produced by spleen cell cultures from mf-infected, but not L3-infected mice. IFN- $\gamma$  has also been shown to be directly involved in effector T cell death, since neutralising anti-IFN- $\gamma$  mAb, but not anti-IL-2, blocked the

death of a Th1 clone induced by ligation of the TCR in the absence of costimulation (Liu and Janeway, 1990). The role of IFN- $\gamma$  in mediating T cell apoptosis induced by infection with mf, could be investigated by measuring apoptosis in mf-infected IFN- $\gamma$  KO mice and/or neutralising IFN- $\gamma$  in spleen cell cultures from mf-infected BALB/c mice. The confirmation of such a role for IFN- $\gamma$  would support the mechanism that apoptosis occurs in mf-infected mice because of insufficient costimulation during antigen presentation.

Another approach to defining the mechanism of T cell apoptosis in mf-infected mice was to investigate the role of Fas/FasL interaction by comparing the response of MRL lpr/lpr (Fas negative) mice versus MRL lpr/C57 (Fas positive) mice to infection with mf. Unfortunately, the results of this experiments was rather confusing, which may relate to the background mouse strain. Infection with mf did not appear to increase the level of apoptosis in freshly isolated spleen cells from either MRL lpr/lpr or MRL lpr/C57 mice as determined by PI staining, although the proportion of hypodiploid cells was slightly, but significantly, higher in spleen cell preparations from both mf-infected and uninfected lpr/lpr compared to both groups of lpr/C57 mice. However, despite the fact that the levels of hypodiploid cells in mf-infected lpr/C57 mice was much lower than that of mf-infected BALB/c mice, DNA fragmentation was present in freshly isolated spleen cells from all four groups (mf-infected and uninfected MRL lpr/lpr and mf-infected and uninfected MRL lpr/C57 mice). Therefore, in this experiment, the DNA fragmentation analysis did not correlate with the PI staining data. However, this experiment was performed once only and perhaps the PI staining procedure did not work efficiently on that occasion. In addition, it is known that spleen cells from uninfected MRL lpr/lpr and lpr/C57 mice undergo spontaneous death in culture much more rapidly than cells from other mouse strains (Van Houten and Budd, 1992).

In an attempt to avoid that particular complication, TUNEL-stained sections from MRL-lpr/lpr mice were also examined. However, a low number of of apoptotic nuclei

were observed dispersed individually throughout the spleen, that was comparable between sections from mf-infected and uninfected mice. However, due to the high background level of apoptotic cells, by PI staining, in uninfected lpr/lpr and lpr/C57 mice, it was not possible to conclude, from this experiment alone, that infection with mf was unable to induce T cell apoptosis in lpr/lpr mice and that, therefore, the mechanism in mf-infected BALB/c mice was Fas-mediated.

Analysis of the proliferative and cytokine responses of lpr/lpr and lpr/C57 mice did clarify the situation somewhat. In comparison to BALB/c mice, spleen cells from both infected and uninfected lpr/lpr and lpr/C57 mice were unable to proliferate normally nor did they produce any IL-2 in response to ConA. In addition, spleen cells from neither lpr/lpr or lpr/C57 mice produced antigen-specific IFN- $\gamma$ . Therefore, on the basis of apoptosis experiments and the cytokine/proliferation experiments together, it appears as if infection with mf has no effect on either lpr/lpr or lpr/C57 mice. It is possible that the proliferative and cytokine responses observed were poor because the conditions used were optimised for BALB/c spleen cell culture. However, perhaps in the absence of antigen-stimulated IFN- $\gamma$  (if that indeed is the mediator) it would not be expected that apoptosis would occur in spleens from either mf-infected lpr/lpr or lpr/C57 mice. Therefore, it may be difficult to use lpr/lpr mice to provide conclusive evidence for the role of Fas, since these mice appear to be double-deficient in both Fas and mf-induced IFN- $\gamma$ . However, when the antigen-stimulated proliferative response of spleen cells from lpr/lpr and lpr/C57 mice are examined, it reveals that at 4 d the S.I., in both mf-infected lpr/lpr and lpr/C57, but not uninfected mice from either group, is  $<1$ . Since this was the original evidence that spleen cells from mf-infected BALBc mice were undergoing apoptosis, then perhaps infection with mf is inducing apoptosis in lpr/lpr and lpr/C57 mice and, therefore, the mechanism is Fas-independent. The next question is how to detect it directly in lpr/lpr mice since freshly isolated spleen cells cannot be used. Since the successful TUNEL experiments in BALB/c mice were performed on spleens from mice infected with  $2 \times 10^5$  mf, then perhaps this higher dose of mf is

required to detect an increase in apoptosis in mf-infected lpr/lpr mice over the high background level present in spleens from uninfected lpr/lpr mice. Alternatively, FasL expression could be examined in spleen sections from mf-infected BALB/c mice to investigate the role of the Fas/FasL interaction indirectly. Depending on the outcome of such experiments it would be interesting to also assay for, and perhaps block, TNF- $\alpha$  in spleen cell cultures from mf-infected mice.

Another interesting angle to studying what factors determine whether or not a T cell dies is to characterise apoptosis-resistant T cells. This idea was based on the observation that while injection of mice with SAg (Kawabe and Ochi, 1991) and injection (Zhang *et al*, 1996; Kearney *et al*, 1994) or feeding (Chen *et al*, 1995) of Tg mice with specific antigen, results in AICD of the majority of antigen-specific cells *in vivo*, a small proportion of antigen-specific T cells escape death and remain in the periphery. A residual population also survives when T cell hybridomas and mature T cells are stimulated to undergo AICD *in vitro* (Lenardo, 1991; Klas *et al* 1993; Wesselborg *et al*, 1993, Dhein *et al*, 1995; Brunner *et al*, 1995; Ju *et al*, 1995). In the study by Zhang *et al* (1996) it was revealed that the only difference that could be detected between antigen-specific T cells that undergo apoptosis and those that survive and, therefore, appear resistant, was the cytokines they expressed. The Tg TCR system used in that study was specifically developed to exclude the possibility that some antigen-specific T cells only persisted after challenge with antigen because they never had contact with the antigen due to the high ratio of antigen-reactive cells:antigen and/or short-half life of administered antigen. Therefore, CD8<sup>+</sup> T cells (from 2C<sub>F1</sub> mice H-2<sup>b/d</sup>, Ld<sup>-</sup>, 1B2<sup>+</sup>) with a Tg TCR reactive against the L<sup>d</sup> MHC I antigen were injected into *scid*<sub>F1</sub> mice (H-2<sup>b/d</sup>) mice expressing L<sup>d</sup> antigen on the surface of all nucleated cells. After 3 days, there was an expansion in 1B2<sup>+</sup>CD8<sup>+</sup> cells in the *scid*<sub>F1</sub> mice accompanied by marked apoptosis, so that by two weeks the majority (~ 80%) of 1B2<sup>+</sup>CD8<sup>+</sup> cells had disappeared from the periphery. The 20-30% of residual 1B2<sup>+</sup>CD8<sup>+</sup> cells were activated (pgp-1<sup>hi</sup>, MEL-14<sup>lo</sup>) and expressed high levels of Fas

and Fas L indicating that the apoptosis-resistant phenotype of these antigen-specific T cells was not due to lack of Fas/FasL interaction. The major distinction was that apoptosis-resistant 1B2<sup>+</sup>CD8<sup>+</sup> cells expressed high levels of IL-4 and IL-10, but not IFN- $\gamma$ , mRNA. In addition, IFN- $\gamma$ , but not IL-4 or IL-10, mRNA was upregulated in 1B2<sup>+</sup>CD8<sup>+</sup> cells at 5 days after injection coincident with the time of maximal apoptosis. Furthermore, co-injection of *scid*<sub>F1</sub> mice with naive 1B2<sup>+</sup>CD8<sup>+</sup> cells and residual 1B2<sup>+</sup>CD8<sup>+</sup> cells, impaired the initial expansion of newly injected antigen-specific T cells .

These data suggested that residual Th2-like apoptosis-resistant antigen-specific T cells may function to maintain peripheral tolerance by suppressing the proliferation of other antigen-specific T cells. Therefore, antigen-specific T cells that undergo apoptosis express IFN- $\gamma$  at the time of maximal apoptosis. In contrast, antigen-specific T cells that are resistant to apoptosis, contain high levels of IL-4 and IL-10, but not IFN- $\gamma$ , mRNA. These findings are consistent with the fact that spleen cells from L3-infected mice, in which there is no evidence of infection-related apoptosis in spleens *in vivo* or in freshly isolated samples, secrete antigen-specific Th2 cytokines IL-4, IL-5 and IL-10, while spleen cells from mf-infected mice produce antigen-specific IFN- $\gamma$ , exclusively (Chapter 3, Expt 1). Furthermore, IL-10 inhibits apoptotic cell death induced in T cells from patients infected with EBV (Taga *et al*, 1994). Both hIL-10 and vIL-10, which are produced by EBV-infected B cells, can promote the survival of T cells that are otherwise destined to die rapidly *in vitro*, perhaps via their ability to inhibit IFN- $\gamma$  production (and IFN- $\gamma$  mediated apoptosis?).

This finding would also be consistent with the fact that Th1 clones appear to be more susceptible to anti-TCR stimulated cell death than Th2 cells (Ramsdell *et al*, 1994). In certain studies this differential ability to undergo AICD correlated with the expression of FasL on Th1 but not Th2 cells (Ramsdell *et al*, 1994) and the presence of FasL mRNA in Th1 cells but not Th2 cells (Almerigogna *et al*, 1996), although in the study

by Zhang *et al* (1996) mentioned above, Fas/FaL expression was comparable on apoptosis supetible and resistant antigen-specific T cells. Therefore, the possible mechanisms controlling the differential susceptibility of Th1 and Th2 cells to apoptosis are open for further investigation and the contrasting properties of spleen cells from L3- and mf-infected mice would make a useful model. It would be interesting to examine whether the expression of *bcl-2* and related genes differs in spleen cells from L3- and mf-infected mice.

The final question is obviously, what the implications are, if any, of mf-related T cell apoptosis in terms of host resistance and protection against pathology in filariasis. If IFN- $\gamma$  does mediate apoptosis and /or Th1 cells are more susceptible to apoptosis than Th2 cells in this model, this may explain why repeated infection of BALB/c mice with live mf or mf extract eventually results in a switch from a Th1 to a Th2 response (Pearlman *et al*, 1993). Since acquired resistance to i.p. challenge with mf correlates with local Th2 responses (Pearlman *et al*, 1993a), then perhaps apoptosis of antigen-specific Th1 cells in mf-infected mice may be a host-directed mechanism aimed at rapidly skewing the immune response in the Th2 direction. A new technique, combining TUNEL with intracellular cytokine staining (Zhang *et al*, 1995), would allow the T cell subset undergoing apoptosis in mf-infected mice to be confirmed. In the human, microfilaraemia is generally associated not only with poor proliferative and IFN- $\gamma$  responses and enhanced IL-4 production in antigen-stimulated PBMC cultures, but also with protection from disease. It is tempting to speculate that the long-term persistence of mf in the blood is directly responsible for this outcome by inducing apoptosis of antigen-reactive Th1 cells. It would be interesting to discover if PBMCs from microfilaraemics display any evidence of apoptotic cell death in culture comparable to that of virally-infected patients described before.

## **CHAPTER 7**



## Chapter 7 Final Discussion

Lymphatic filariasis is a complex disease of high morbidity affecting 118 million people worldwide. *W. bancrofti*, *B. malayi* and *B. timori* L3 enter via the skin and mature into adult worms that live within the afferent lymphatics for up to five years and mate to release millions of mf into the bloodstream. The spectrum of infection and immune responses is broad, ranging from an asymptomatic microfilaraemic state, to those suffering from the lymphatic pathology, such as chronic lymphodema and elephantiasis, that are characteristic of the disease. Originally, microfilaraemics were considered to be "immunosuppressed", while chronic pathology patients were identified as "hyper-reactive" (Ottesen *et al*, 1977; Piessens *et al*, 1980; Nutman *et al*, 1987; Nutman *et al*, 1987a). However, these early observations were based on proliferation data and used small study groups. The detection of parasite-specific IgG4 has since proved to be a useful indicator of active infection especially in cases where there is no outward signs of infection and/or disease (Kwan-Lim *et al*, 1990).

It has subsequently been demonstrated that actively infected humans across the clinical spectrum, who are by definition exposed to all the life cycle stages, generally exhibit an antigen-specific proliferative defect but generate strong Th2 responses (Yazdanbakhsh *et al*, 1993; Yazdnabakhsh *et al*, 1993a). The Th2 response is characterised by an increase, relative to non-endemics, of CD4<sup>+</sup> T cells producing IL-4 and IL-5 in response to filarial antigen, serum IgG4 and IgE and peripheral blood eosinophilia (Mahanty *et al*, 1993). The inability of PBMCs to proliferate or secrete IFN- $\gamma$  in response to antigen has been defined as a selective Th1 defect (King *et al*, 1993, Maizels *et al*, 1995).

A few recombinant proteins from *Brugia* (Paxton *et al*, 1993) and *Onchocerca* (Gurraud *et al*, 1995) have been identified that may be capable of stimulating Th2-like responses. However, as yet, the pathways and cell types that are responsible for Th2 polarisation in

the infected human have not been investigated. In contrast, a number of mechanisms have been suggested to give rise to Th1 unresponsiveness including clonal deletion, anergy, and suppression. A few studies have implicated the induction of IL-10 (King *et al*, 1993) and certain parasite products for mediating suppression (Piessens *et al*, 1980; Lal *et al*, 1990; Liu *et al*, 1992). In addition, infected individuals possess diminished levels of antigen-responsive T cells in their peripheral blood (King *et al*, 1992) consistent with a mechanism of T cell anergy and/or deletion, although there has been no direct evidence that parasite-induced anergy or clonal deletion actually occurs in humans. However, the concept of clonal deletion is a persuasive one, based as it is on the observations that children born of *W. bancrofti* microfilaraemic mothers are more likely to become microfilaraemic (Lammie *et al*, 1991) and exhibit long-term proliferative unresponsiveness (Steel *et al*, 1994) than children born of amicrofilaraemic mothers, while paternal status has no effect on the outcome of infection. Furthermore, it is known that non-endemic persons develop disease more rapidly after infection than is the average for the endemic population (Partono *et al*, 1978).

Studying the responses of infected individuals, from the different clinical categories, following DEC treatment has been particularly useful in demonstrating that more than one distinct mechanism may account for T cell unresponsiveness in individuals infected with brugian filariasis. Antigen-stimulated proliferation and IFN- $\gamma$  release were elevated after long-term DEC treatment of infected persons who had been non-responders before chemotherapy (Sartono *et al*, 1995), indicating that alternative, non-deletion mechanisms must exist. Furthermore, the responses were more substantially enhanced in asymptomatic microfilaraemics and elephantiasis patients than in microfilaraemics, indicating that additional mechanisms of suppression are associated with the presence of microfilariae. Similarly, unresponsiveness in patients with elephantiasis was more easily recovered by *in vitro* treatments than in microfilaraemics and asymptomatic amicrofilaraemics (Sartono *et al*, 1995a). However, unresponsiveness was irreversible following *in vitro* manipulation in the majority of patients from all clinical categories.

Therefore, an important conclusion from these recent studies is that not only are life-cycle stages other than the mf involved in the down-regulation of Th1 responses in the infected human, but that different stages of the parasite may use distinct mechanisms.

The factors complicating the study of human filariasis includes not only *in utero* exposure, but also differences in transmission levels, HLA type and co-infection with other pathogens. In addition, since certain responses can be observed in individuals across the clinical spectrum of infection, the immunological mechanisms underlying immunity and disease in man are extremely difficult to interpret. Furthermore, the fact that lymphatic filariae parasitize the centre of the immune system, in a manner similar to many intracellular organisms which invade the very cells of the immune system that are supposed to be involved in their destruction, means that the host is not able to unleash destructive immune responses that may be permitted elsewhere in the body. Therefore, the down-regulated immune state may be as much host-mediated as parasite-mediated, thus reflecting a highly evolved and complex host-parasite relationship.

The initial aim of this study was to characterise the immunomodulatory potential of *B. pahangi* L3 using a mouse model. This is an area that had previously been overlooked in favour of analyzing immunomodulation by adults (Th2 polarization) and mf (early Th1 response) (Lawrence *et al*, 1994). Based on the evidence from human and animal studies, that naturally occurring immunity is targetted at L3 and that proliferative unresponsiveness was associated with microfilaraemia, the focus of earlier studies had been on analysing the mechanisms of anti-L3 immunity. Mice that had been immunised with irradiated L3 were protected against challenge infection and generated a Th2 response (Bancroft *et al*, 1993). Therefore, in initial experiments mice were given a primary s.c. infection with normal L3. Mf were included primarily as a control group for "intact Th1 responses", although it soon became apparent that this stage, injected s.c., was capable of a novel form of immunomodulation. The experiments demonstrate that primary single stage infections of BALB/c mice with L3 and mf appear to replicate

the two prominent features of the human immune response. L3 induce an exclusive Th2 response such that normal Th1 functions are suppressed, while spleen cells from mf-infected mice are unable to proliferate in response to antigen but retain the capacity to produce cytokines.

Primary infection with L3, via the s.c. (natural) route, resulted in a Th2 polarised response characterised by elevated levels of IL-4, IL-5 and IL-10 in spleen cell cultures in response to ConA and antigen and the production of parasite-specific serum IgG, IgG1 and IgE, but not IgG2a. In contrast, no IL-2 or IFN- $\gamma$  could be detected in antigen-stimulated cultures. In addition, ConA-driven proliferation, IL-2 and IFN- $\gamma$  production of spleen cells from L3-infected mice were drastically reduced compared to that of cells from uninfected controls, but could be restored by incubating the cells with neutralising anti-IL-4 or anti-IL-10 MAbs. These results indicate that a profound level of Th2-mediated suppression exists in L3-infected mice that may extend to other antigens. This is consistent with the finding that responses to non-filarial antigens in infected humans may also be impaired; treatment with filaricidal drugs significantly enhances the proliferative response to PPD in individuals infected with lymphatic filariae (Sartono *et al*, 1995) or *O. volvulus* (Soboslay *et al*, 1994). Furthermore, it has recently been observed that antigen, mitogen and PPD (a Th1-type antigen) responses are severely impaired in *W. bancrofti*-infected individuals living in areas of very high transmission (C. King pers. comm.). Administration of PPD to mice in which a Th2 response has been established, i.e. over 3 weeks after i.p. inoculation of mice with *B. malayi* mf or multiply immunisations with mf extract (Pearlman *et al*, 1993b), skews the subsequent response to PPD in the Th2 direction (IL-4 and IL-5 were produced in addition to IFN- $\gamma$ ) (Pearlman *et al*, 1993b). In contrast, in one human study the cytokine profile of PBMCs from actively infected individuals in response to PPD is unaltered (Sartono *et al*, 1996). The discrepancy in these results may be attributable to a much higher parasite dose in the experimental model. However, in view of the data that infection with high

doses of L3 is able to impair polyclonal responses in mouse and man, it might still be worth analyzing the response of mice given both L3 and PPD.

The route of infection was also shown to play a role in determining the Th cell response to L3, as had previously been demonstrated with simple antigens (Aichele *et al*, 1995). A strong Th2 cell response was generated and Th1 responses downregulated when L3 were given s.c. but not i.p. This result also suggested a role for APC in biasing the Th cell response to infection with L3. In fact replacing the APC population from the spleens of L3-infected mice restored the polyclonal Th1 cell responses. However, it remains to be determined whether APCs downregulate Th1 responses via secreted and surface co-stimulatory/inhibitory functions and/or IL-4 and IL-10 are operating via their effect on APC.

Another original finding was that neutralising IL-10, but not IL-4, in spleen cell cultures from L3-infected mice permitted the expression of antigen-specific Th1 cytokines, indicating that *Brugia*-reactive Th1 cells are indeed primed, but suppressed, following infection with L3. Previously the interpretation of the finding that IL-4 KO mice infected with L3 did not produce antigen-specific IL-2 and IFN- $\gamma$ , had been that L3 do not possess any Th1-stimulating antigens (Lawrence *et al*, 1995). Obvious future experiments would be to analyze the response of IL-10 KO mice infected with L3, and to further investigate the mechanism by which IL-10 suppresses antigen-specific Th1 cells.

The analysis of early cytokine mRNA expression in the draining LN from L3-infected mice was also consistent with the active suppression of antigen-specific Th1 activity. The response to L3 was polarised in the Th2 direction from the outset, since the levels of IL-4 and IL-10 mRNA were elevated in the CD4<sup>+</sup> population at day 4 p.i., in the absence of IFN- $\gamma$  expression. However, the most striking result of this analysis was that L3 induce a burst of IL-4 mRNA expression at 24h p.i. from an unusual CD4<sup>+</sup>CD8<sup>-</sup>

(DN) T cell population. The promptness of the response suggests that the DN T cells are recognising non-polymorphic L3 antigens, that may be lipid in nature by analogy with DN T cells that are reactive against mycobacterial lipids (Beckmann *et al*, 1994, Sieling *et al*, 1995). One possibility is that IL-4 producing DN T cells have evolved to function, following filarial infection (and other nematode infections that are initiated via the skin?), by bridging innate and adaptive immune systems in a manner akin to macrophages in bacterial infections. In addition, the fact that the mycobacteria-reactive DN T cells were obtained from skin samples of normal and leprosy patients may suggest that L3-reactive DN T cells are not simply restricted to mice. Clearly further experiments are necessary to define the IL-4 producing DN T cell population and the nature of their target antigen(s) more accurately and to assess whether their activation is an absolute requirement for the L3-induced Th2 response.

The ability of s.c. infection with *B. pahangi* mf to induce a very different response than L3, stimulating spleen cells to secrete antigen-specific IFN- $\gamma$  and the production of serum IgG2a, was consistent with previous results, early after i.p. infection with *B. malayi* mf (Lawrence *et al*, 1994). Microfilaraemics express the lowest levels of IgE in comparison to other infected groups (Kurniawan *et al*, 1993), an isotype known to be exquisitely sensitive to inhibition by IFN- $\gamma$  (Ishizaka *et al*, 1990). In this context, IFN- $\gamma$  produced in response to mf may act to modulate strong Th2 response stimulated by L3 and adult worms. While a Th2 response clearly predominates in mice infected i.p. with mf producing female worms (Lawrence *et al*, 1994), mf are confined within the same compartment as the females, whereas in the actively infected human, mf are released into the circulation and may influence peripheral T/B cell responses independently of L3 and adult worms that home to and then remain within the lymphatics for their lifetime. It may be interesting to examine which response predominates in the mouse when both L3 and mf are given by their natural routes.

However, the original findings in the experiments with mf, was that spleen cells from mice infected s.c. with mf are unable to proliferate in response to antigen and in fact die

rapidly when cultured in the presence of antigen. While the uncoupling of proliferation and cytokine production was suggestive of anergy induction, further analysis revealed that spleen cells, most likely T cells, from mf-infected mice were undergoing apoptosis. This was evidenced by the presence of fragmented DNA and an increased number of lymphocytes with hypodiploid DNA content in freshly isolated spleen cells from mf-infected animals. Further evidence of apoptosis was provided by the detection of TUNEL stained cells within the T cell areas of the follicles in spleen sections from mf-infected mice but not L3-infected mice or uninfected controls. In addition, spleens from mice infected i.v. with mf exhibited similar clusters of TUNEL stained cells as s.c. infected mice, suggesting that mf-induced T cell deletion, by apoptosis, may occur during natural infection. However, while it is likely that T cells are the cell type undergoing apoptosis in mf-infected mice, further experiments are required to demonstrate this conclusively.

One obvious mediator of mf-induced apoptosis is IFN- $\gamma$  since it is present in high levels in antigen-stimulated spleen cell cultures from these mice and has been shown to be directly involved in the apoptosis of a Th1 clone activated in the absence of costimulation (Liu and Janeway, 1990). Furthermore, like other examples of apoptosis-resistant cells (Van Parijs *et al*, 1996; Zhang *et al*, 1996), spleen cells from L3-infected mice secrete Th2 cytokines IL-4, IL-5 and IL-10 and not IFN- $\gamma$  in response to antigen. However, IFN- $\gamma$  is not itself sufficient for T cell death, since large amounts of IFN- $\gamma$  are produced when the Th1 clone is stimulated with anti-TCR MAb and accessory cells, which does not cause cell death (Liu and Janeway, 1990). Therefore, other survival factors and/or signals (i.e. costimulatory signals) must be acting when T cells are functionally activated. CD28 costimulation has been shown to augment T cell survival during antigen activation by enhancing expression of bcl-x<sub>L</sub> (Boise *et al*, 1993). The effect of costimulation, or more correctly the lack of it, in inducing apoptosis in mf-infected mice is worth further investigation, especially since preliminary experiments in *lpr* mice suggests that the response is Fas-independent. In addition, it would be

interesting to determine whether any differences in intrinsic cell survival factors, like *bcl-x<sub>L</sub>*, can be detected between spleen cells from mice infected with L3 or mf.

The ability of an extracellular parasite, like mf, to induce host cell apoptosis should not be that surprising considering that the pathway of apoptosis is so highly conserved. In fact, the nematode *Caenorhabditis elegans* provided the first model system in which the early stages of PCD were observed. Using embryonic mutants, a number of genes (*ced* genes=*C. elegans* death genes) were discovered to encode for proteins that implement PCD. Members of the *bcl-2* and ICE family of proteins have now been revealed to be the mammalian homologues of these proteins. Of the 1090 somatic cells generated during development of *C. elegans*, 131 are destined to die. Their suicide requires the expression of *ced-3* and *ced-4*, and can be blocked by expression of *ced-9* (Hengartner *et al*, 1992). Not surprisingly, *ced-9* has recently been shown to be homologous to the *bcl-2* family (Hengartner and Horvitz, 1994). While the sequence of *ced-4* has provided no clues as yet to its function, *ced-3* appears to be most homologous to the ICE-like protease CPP32 (Fernandes-Alnemri *et al*, 1994). Significantly, over-expression of *ced-3* (or ICE) in mammalian fibroblasts results in apoptosis, that can be blocked by *bcl-2* (Miura *et al*, 1993) while expression of human *bcl-2* inhibits cell death in *C. elegans* (Hengartner *et al*, 1992). Therefore, certain genes, controlling apoptosis of mammalian and nematode cells, are not only structurally, but also functionally, homologous.

The commonality of the intracellular pathways of apoptosis between mammalian and nematode cells, therefore, supports the concept that a parasitic nematode like *Brugia* may have evolved to develop extracellular triggers of host cell apoptosis. This may rely indirectly on infection with mf down-regulating costimulatory functions and stimulating the host cells to secrete factors like IFN- $\gamma$  or TNF- $\alpha$ , or upregulate FasL, to induce their own suicide. Alternatively, mf may produce homologues of these cytokines or soluble homologues of FasL to directly induce apoptosis of activated host T cells. If the full extent of this hypothesis is accurate then, presumably, the parasite would not express



the common receptors that could signal death or would express a form of these receptors that could not be used by mammalian products. In addition, the parasite is bounded by an extracellular cuticle and this may provide protection against damage to its own cells. Conversely, since Th1 cells appear to be more susceptible to apoptosis than Th2 cells, the induction of host T cell apoptosis following infection with mf, may simply represent an immune deviation mechanism used by the host to generate a protective Th2 response. Whatever the driving force behind it, it seems likely that induction of host cell apoptosis in mf-infected mice has important implications for modulating the host response to the parasite. The possible involvement of apoptosis-related TNF- $\alpha$  in host protection and parasite survival in schistosomiasis is similarly complex. TNF- $\alpha$  has been implicated in protection against infection with schistosomula (Hagan *et al*, 1993), perhaps by inducing parasite apoptosis (Garside *et al*, 1996a). However, TNF- $\alpha$  also triggers egg laying and is thought to be necessary for continued transmission by stimulating granuloma formation which may assist eggs to escape from tissues (Hagan *et al*, 1993).

In relation to the human disease of lymphatic filariasis, the discovery that infection with mf can induce T cell apoptosis would provide a mechanism to support the concept of clonal deletion. A further implication of these results is that clonal deletion is not restricted to *in utero* exposure but may reflect a more general mechanism by which peripheral tolerance is induced by mf in the infected child or adult. Such an additional mechanism of deletion by mf may explain why antigen-stimulated proliferation was more substantially enhanced after long-term DEC treatment of asymptomatic microfilaraemics and elephantiasis patients than microfilaraemics.

The use of this mouse model to independently dissect the primary responses to L3 and mf has uncovered several novel mechanisms by which filarial parasites may modulate the immune system of their human hosts. The results have also provided some insight

into how the intimate relationship between T cell activation, anergy and death is regulated in the immune response towards a single parasite.

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